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COMMISSION ON EPIDEMIOLOGICAL SURVEY

ANNUAL REPORT TO THE ARMED FORCES EPIDEMIOLOGICAL BOARD

FISCAL YEAR 1966

MARCH 1967

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MARCH 1967

SUMMARY

Progress is reported in selected areas of research in medical defense aspects of biological agents by the U. S. Army Medical Unit and one contractor.

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COMMISSION ON EPIDEMIOLOGICAL SURVEY

MEMBERS

Cluff, Leighton E., Jr., M.D.

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Woodward, Theodore E., M.D., Director

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McCrumb, Fred R., Jr., M.D.

Wiseman, Charles L., Jr., M.D.

THE DIRECTOR'S SUMMARY REPORT

The Commission on Epidemiological Survey held its annual meeting at the Walter Reed Army Institute of Research on 9 September 1966. Senior representatives of the Department of Army, Navy and Air Force and the U. S. Army Medical Unit and other personnel at Fort Detrick who attended were:

U. S. Army Medical Unit

Colonel W. R. Beisel, MC
Captain M. K. Ward, USPHS
Lt Colonel K. R. Dirks, MC
Lt Colonel R. W. McKinney, MSC
Major M. I. Rapoport, MC
Major G. E. Shambaugh, III, MC
Captain A. C. Alevizatos, MC
Captain C. P. Craig, MC
Captain R. D. Feigin, MC
Captain E. V. Staab, MC
Dr. G. Lust
Dr. V. G. McGann

U. S. Army

Colonel J. W. Cooch, MC, Walter Reed Army Institute of Research
Colonel L. P. Frick, MSC, Medical Research & Development Command
Colonel G. W. Johnston, MSC, Medical Research & Development Command
Colonel R. M. Nims, VC, Fort Detrick
Lt Colonel R. T. Cutting, MC, Medical Research & Development Command
Lt Colonel J. Einarson, MC, Office of The Surgeon General
Lt Colonel J. C. Fitzpatrick, MC, Medical Research & Development Command

U. S. Navy

Captain J. W. Millar, Bureau Medicine and Surgery
Commander L. W. Miller, Bureau Medicine and Surgery

U. S. Air Force

Colonel S. Lutz, Jr., MC, HQ, USAF, Office of the Surgeon General

Guests

Dr. Frank A. Carozza, Jr., Union Memorial Hospital, Baltimore, Maryland
Dr. Harold N. Glassman, Fort Detrick
Dr. Riley D. Housewright, Fort Detrick

Dr. Gustave Dammin, President of the Armed Forces Epidemiological Board, attended and made many helpful suggestions in connection with our mission. The Director expressed grateful appreciation to Captain Sidney A. Britten, USN, Executive Secretary of the Board, and to Miss Betty Gilbert, Administrative Assistant, for their continued assistance in conducting the Commission's activities.

The agenda of the one day's meeting was devoted to reviewing work completed or in progress by investigators of the U. S. Army Medical Unit and one of its contractors.

VENEZUELAN EQUINE ENCEPHALOMYELITIS VACCINE

Clinical studies of 40 persons given attenuated Venezuelan equine encephalomyelitis (VEE) vaccine revealed demonstrable viremia in 13 subjects and clinical symptomatology in 15 subjects. The clinical findings did not correlate with the presence of viremia. Transient electrocardiographic abnormalities were noted in 47.5% and transient leukopenia in 40% of the vaccinees. Eight individuals followed with daily electroencephalograms demonstrated no significant change subsequent to vaccination. There was no statistically significant correlation between any of these responses or combination of responses. The virologic studies demonstrated the presence of low grade viremia up to day 12 postinoculation. In view of the low level of viremia it appeared unlikely that vaccinees would infect Aedes aegypti mosquitoes. Members of the Commission on Viral Infections kindly studied the data and reached the same conclusion. Further studies are in progress.

METABOLIC CHANGES IN INFECTIOUS DISEASES

Intensive studies of metabolic and biochemical changes occurring in man during the incubation period and clinical course of various infectious diseases have been conducted. The objective of such appraisals has been to develop a better understanding of the fundamental nature of infectious processes and to search for objective clues which might lead to early diagnosis. In tularemic infection caused by aerosolization of virulent Pasteurella tularensis (SCHU-S strain) levels of certain blood amino acids are decreased from 12-36 hr before the onset of fever. The changes are greater in those persons with more severe illness. The loss does not result from excess urinary loss but perhaps from excess utilization by the liver and spleen. Amino acids are used for new protein synthesis.

There is a diurnal change in the concentration of blood amino acids in normal individuals. Following VEE vaccine administration at 0800 hr, there occurred an obliteration of the normal diurnal amino acid rhythm that began within 1 day and persisted for 4 days. When the same vaccine was administered to 20 other volunteers at 2000 hr, there was an early rise in blood amino acids of 2 days duration followed in turn by a fall to below normal concentrations for a total of 6 additional days. The changes in this latter group also included a loss of the diurnal rhythm,

but in addition several patients showed up to an 80-fold increase in proline, a marked increase in glutamic acid, and a depression in glutamine. Thus, the exposure at 2000 hr was accompanied by a distinct pattern of metabolic changes, some of which might involve an inhibition of the enzyme glutamine synthethase.

Changes in another enzyme, tryptophan pyrrolase were studied in the livers of mice infected with a small number of pneumococci. This infection was associated with a stimulation in the activity of this enzyme within 2 hr, but late in the infection the enzyme activity showed depression as it does during endotoxemia. The early induction of tryptophan pyrrolase was dependent upon the presence of an intact adrenal gland, an observation compatible with the known ability of cortisol to induce this enzyme. Late in infection, however, when plasma steroid levels were high, the enzyme activity fell and could not be stimulated by additional cortisol, an observation compatible with toxic inhibition of enzyme synthesis in a dying animal. Incidental to this study was the first description of a circadian change in this enzyme.

The infection-related stimulation of synthesis of new protein molecules within host cells is initiated by early sequential changes in deoxyribonucleic acid and ribonucleic acid (RNA) function within the cell. These were studied in several tissues of the intact mouse after infection with either Diplococcus pneumoniae or the Trinidad strain of VEE; similar studies with VEE were conducted in cultured tissue cells. It was possible to show differences in the direction of change in RNA and protein metabolism due to either the bacterial or viral infection. In addition, VEE infection of cultured cells was shown for the first time to be associated with induction of a new viral RNA synthetase; present techniques have not permitted identification of this enzyme in the intact animal host.

Studies of staphylococcal enterotoxin reveal that it is highly antigenic although the rate of antibody formation is slow. There appears to be a relationship between the level of circulating antibody and degree of resistance to enterotoxin in primates. Staphylococcal enterotoxin differs from bacterial endotoxins since pyrogenic tolerance as measured by reticuloendothelial system reactivity is transient. When animals are pyrogenically refractory they react much less to intradermal injection of enterotoxin These studies have broad implications and suggest that antibody inhibits skin reactivity.

The Commission, through the University of Maryland contract, has extended the studies relating to the protective efficacy of typhoid vaccine and pathogenesis of the disease. Typhoid vaccine K (acetone preserved) and L (phenol inactivated) protected volunteers against a low infectious challenge of 100,000 bacilli (expected to produce illness in 25% - ID_{25}). Aerosol exposure to an ID_{25} of Salmonella typhosa failed to induce illness. This suggests that the respiratory tract is not a site of invasion. Penetration through pharyngeal or tonsillar tissue appears to be an unlikely mode of entry. Typhoid bacilli survive for 45 min or more in the stomach.

Organisms apparently multiply in the intestinal lumen and penetrate the mucosa. The lamina propria of the small intestine shows mild inflammatory changes during the incubation period after large oral inocula. Minimal changes occur after an ID_{25} dose. Bacterial interference as a human host defense mechanism has been shown by studies with antibiotics given before infectious challenge. Nonabsorbable antibiotics reduce the numbers of intestinal bacteria altering the pH. Under these circumstances smaller numbers of bacilli cause clinical illness. Further interference studies are in progress.

The extensive studies of the effect of bacterial endotoxin in animals and human subjects show that during active infection with S. typhosa man acquires increasing hypersensitivity to the endotoxic component of the microbe. Intradermal testing with purified S. typhosa during illness reveals intensified 24-hr inflammatory lesions when compared to control preinfection responses and intravenous injection of the endotoxin elicits markedly hyperreactive pyrogenic responses and subjective toxic responses. The endotoxin tolerance mechanisms possessed by normal man remain intact. The repeated administration of endotoxin intermittently or by continuous intravenous infusion causes tolerance to develop rapidly within the framework of the hyperreactive state. In spite of marked activation of these tolerance mechanisms, the febrile and toxic course of the disease remains unchanged indicating that endotoxemia does not account for the sustained illness. Although endotoxemia may not account for sustained illness, the release of a relatively small bolus of endotoxin into the circulation during typhoid fever (or upon institution of appropriate antibiotic therapy), could readily induce an abrupt intensification of the febrile and toxic state in the hypersensitive host.

The vaccine trials indicate that the conventional typhoid vaccines are effective in protecting man against low infecting doses which might be expected in a water-borne exposure but would provide no protection from an exposure resulting from the consumption of heavily contaminated food containing more than 10,000,000 viable typhoid bacilli. Apparently the Vi antigen per se is not responsible for stimulating the protective antibody. Mechanisms involved in vaccine-induced resistance are not understood.

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Resources, National Academy of Sciences-National Research Council.

In these studies the strictest standards which apply to human subjects as volunteers were adhered to.

The Executive Meeting was devoted to hearing of the progress in the staphylococal enterotoxin B program and the need for evaluating several vaccines; VEE, EV plague, and Rocky Mountain spotted fever. The spotted fever vaccine studies will be conducted in collaboration with the Commission on Rickettsial Diseases.

Colonel Dan Crozier presented a paper describing the program of this Commission to the Association of Military Surgeons on 9 November 1966. The 1967 meeting of the Commission, scheduled for 7 and 8 September, will focus on a discussion of metabolic changes in infectious diseases.

Theodore E. Woodward, M.D.
Director
Commission on Epidemiological Survey

CLINICAL STUDIES OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VACCINE STUDIES IN MAN

Aristides C. Alevizatos, Captain, MC*

This project was designed primarily for the purpose of investigating the possible secondary transmissibility of attenuated Venezuelan equine encephalomyelitis (VEE) virus. While carrying this objective there was opportunity also to study in detail for the first time the clinical effects of this attenuated strain of VEE virus in man. Although it has been used routinely for immunization of at-risk personnel, the clinical syndrome it causes had heretofore not been studied in a carefully documented fashion.

Forty young healthy male volunteers without prior VEE virus experience were inoculated on day 0 with 5,000 guinea pig median intraperitoneal infectious doses ($GPIPID_{50}$) of live attenuated VEE virus vaccine (Lot TC 83/3-2 L3). Clinical evaluation of the patients was accomplished at least twice daily throughout hospitalization. Appropriate laboratory studies including electrocardiograms (EKG) were performed daily on all subjects. Daily electroencephalograms (EEG) were obtained on 8 of the 40. Heparinized blood for virus assay was drawn from each man at 12-hr intervals and frozen. Blood for hemagglutination inhibiting (HI) antibody titer against VEE virus was drawn from each man daily as well as on days 28 and 45, and frozen for testing at a later date. Results of the viral and serologic studies are discussed in the next paper.

Incubation time of reaction was measured from time of vaccination to time of onset of clinical response as determined by a sustained temperature rise to > 100 F rectally. There were two distinct incubation periods, one early, ranging from 12-64 hr postvaccination, the other late, averaging 8 days.

Table I represents degrees of the responses in the 40 vaccinees.

TABLE I. DEGREE OF RESPONSE IN 40 VACCINEES

NONE	1+	2+	3+	4+
25	1	6	4	0

Twenty-five demonstrated no clinical symptomatology. Among the remaining 15 subjects, reaction to vaccination was influenza-like, consisting of

* U. S. Army Medical Unit.

headache, myalgia, and malaise as well as sporadic complaints of sore throat, nausea, and anorexia. There was a paucity of significant physical findings. The symptomatic response to vaccination in these 15 was arbitrarily graded on a scale of 1+ to 4+. None warranted a 4+ rating. Four warranted a 3+ rating. Though these 4 men preferred to remain in bed and were quite uncomfortable, their picture was not one of toxicity such as is seen in volunteers ill with tularemia. The duration of their symptoms was approximately 36 hr; recovery was characterized by a rapid rebound to usual states of health. Six received a 2+ rating and 5 rated 1+. Those with 1+ continued their usual activities without difficulty.

The rectal temperature exceeded 101 F in 14 vaccinees and usually correlated in time with the subjective symptomatology. Duration of temperature elevation ranged from 4-72 hr with an average of 22½. Only symptomatic treatment was given. Recovery was spontaneous and without complication in all subjects. Of interest is the finding that 5 subjects experienced a diphasic type of reaction, with clinical response occurring in the early postvaccination period and again during the later peak period of onset of response. The second of these two responses was usually the more marked.

Ancillary laboratory studies are shown in Table II and were performed daily on all subjects. The EEG data revealed no significant abnormalities. One person who had an abnormal baseline had transient changes which returned to his own baseline pattern. The amino acid findings are discussed in a later paper by Dr. Feigin.

TABLE II. ANCILLARY LABORATORY STUDIES

Hemoglobin	Alkaline phosphatase
Hematocrit	Serum glutamic oxalacetic transaminase
Sedimentation rate	Serum lactic dehydrogenase
C-reactive protein	Serum bilirubin
WBC and differential	Creatinine

Amino acid analysis
Electrocardiogram
Chest x-ray (days 2 and 7)
Electroencephalograms (daily on 8 subjects)

With the exception of the white blood cell counts and EKGs, all laboratory studies were normal.

A transient leukopenia (defined as less than 4,000 WBC/cmm) was demonstrated in 16 vaccinees. This was predominately a neutropenia which spontaneously returned to normal within several days in all cases. There was no correlation between clinical reaction, fever or viremia, and the leukopenia. That is, some subjects who were febrile, clinically symptomatic,

with or without demonstrable viremia and manifested leukopenia, while others were as ill with or without demonstrable viremia and did not manifest leukopenia. Others without clinical response, fever or demonstrable viremia, did manifest leukopenia.

Transient EKG changes were noted in 19 of 40 vaccinees. These changes were diminution or inversion of the T wave of the complex, and interestingly enough were invariably found in standard leads 2 and 3, lead AVF, and occasionally in the lateral precordial leads. In Figure 1 are shown selected tracing of the EKG of TWC, a patient whose response clinically rated 2+ and who had fever up to 102.6 F but did not manifest demonstrable viremia. Day -1 represents the preinoculation tracing. The second tracing demonstrates the maximum alteration of his EKG pattern, with an eventual return to the original pattern as shown in the third tracing.

Figure 2 presents selected EKG tracing of another patient, GRC, who was without clinical symptomatology or fever, but who had demonstrable viremia. Again we note changes in the T waves in the inferior and lateral leads, with return to the original pattern. In all cases these changes were transient and were usually of 3-5 days duration. Again there was no positive or negative correlation to clinical symptoms, fever or demonstrable viremia. Eight of these 19 subjects with EKG changes were never clinically symptomatic or febrile.

Viremia was demonstrable in 13 of the 40 vaccinees. The magnitude and epidemiological significance of those with viremia is discussed in the paper which follows. Table III is a correlative resume of those 13 men having

TABLE III. FINDINGS IN SUBJECTS WITH DEMONSTRABLE VIREMIA

SUBJECT	DAY OF VIREMIA	DAY OF MAXIMUM REACTION	DEGREE OF REACTION	MAXIMUM FEVER °F	DAY OF EKG CHANGE	DAY OF LEUKOPENIA
GRC	6,7,8	-	0	-	-	-
WRF	3,4,5,6	4	2+	103.2	5	-
HGG	6	6	3+	104.4	-	4,8
DLG	8	-	0	-	3	-
DPG	3,4,5,6,7	2,4	2+	101.0	-	-
RCH	7	4	2+	103.2	1	-
WLL	7,8	10	2+	104.6	3	5
FHM	2,8,9	-	0	-	-	-
RR	4	2	1+	101.0	-	-
DVR	6	1,7	3+	102.8	1	3,5
DCS	12	2,12	3+	104.2	12	-
RCW	7	-	0	-	2	3,5
JDW	4	-	0	-	2	3,5

TWC 21 W ♂

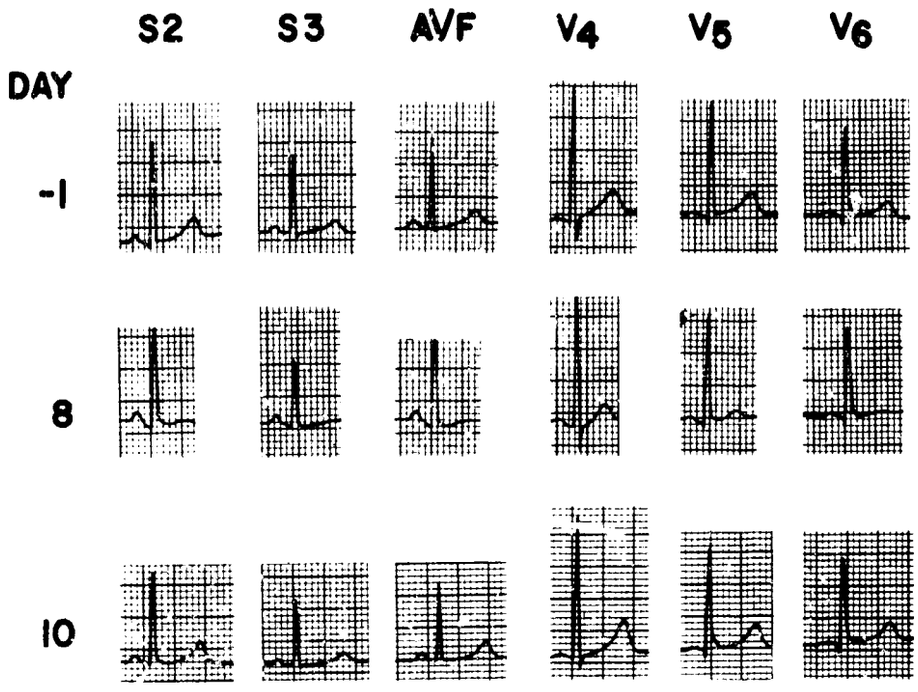

FIGURE 1. SELECTED EEG TRACINGS OF PATIENT TWC.

GRC 26 W ♂

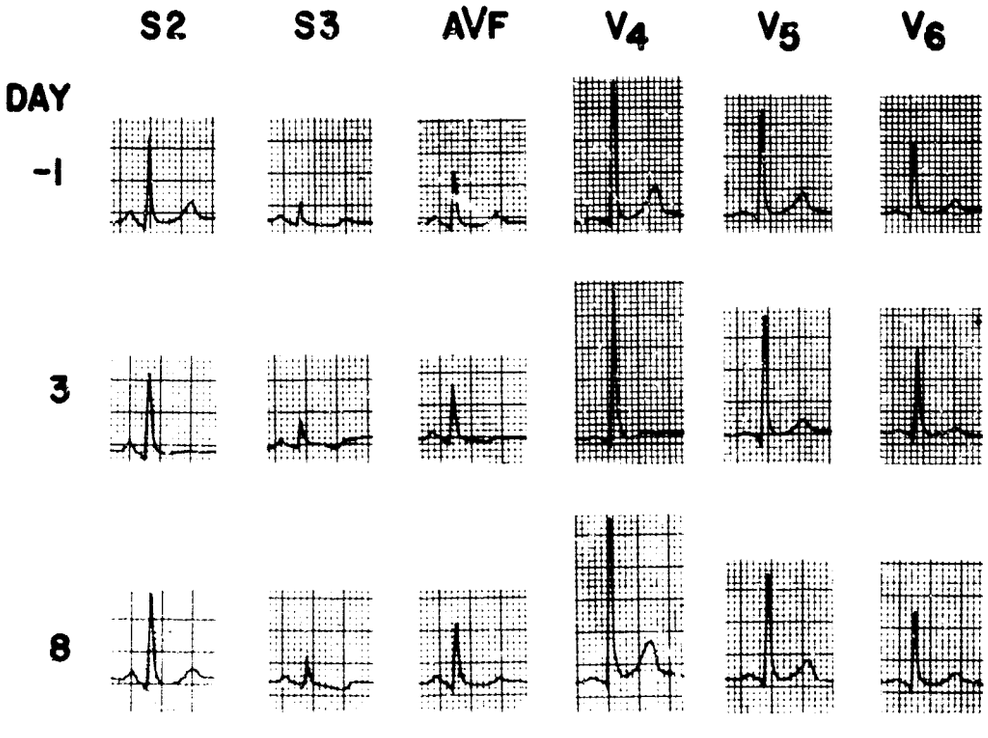

FIGURE 2. SELECTED EEG TRACINGS OF PATIENT GRC.

demonstrable viremia. Note that 5 of these 13 with demonstrable viremia had no significant symptoms or fever, and only 8 of those 15 subjects with symptomatic responses to vaccination had demonstrable viremia. The earliest demonstrable viremia was at 60 hr, while the most distant in time was 12 days postvaccination. Several subjects had viremia demonstrable on multiple occasions and the total number of positive blood specimens from these 13 subjects was 28. Again as can be seen here, there was no correlation between symptomatology or fever and demonstrable viremia. For example, GRC has 4 positive blood specimens for viremia (2 on day 7) but manifested no symptoms or fever, and likewise had no EKG changes or leukopenia. HGG had symptoms and fever at the time of his demonstrable viremia, but his leukopenia did not correlate in time with his viremia. DPG had symptoms during his viremia but continued to manifest viremia after his clinical symptomatology had resolved. WLL however had no symptoms until 2 days after his demonstrable viremia. DCS is of interest in that his viremia was the most distant in time of those demonstrated. Also he was the only one of those who experienced a diphasic reaction whose first episode was more marked in symptomatology than the second.

Thus again there was no correlation between clinical response or fever, and demonstrable viremia.

SUMMARY

Forty volunteers were immunized with live attenuated VEE virus and evaluated closely from clinical, laboratory, virologic, and serologic standpoints. Some degree of reaction was noted in 37.5% of these individuals with 10% of them having 3+ reaction. Viremia was demonstrable in 32.5%; 47.5% had transinet EKG abnormalities; 40% had transient leukopenia. There was no consistent positive or negative correlation between any of these responses or combination of responses.

VENEZUELAN EQUINE ENCEPHALOMYELITIS VACCINE VIREMIA STUDIES IN MAN

Robert W. McKinney, Lt Colonel, MSC*

As has been indicated in previous reports to this Commission,[1,2] use of the live attenuated Venezuelan equine encephalomyelitis (VEE) vaccine for immunization of man has been restricted to periods when mosquitoes are absent from the environment. This restriction was established because of the demonstrated capacity of the virus to regain virulence on serial intracerebral passage in mice and because of evidence that viremia occurred in man following inoculation of the vaccine.

Before presenting the results of a recent study designed to determine the duration and level of viremia in persons inoculated with the vaccine results of earlier studies bearing on this question are reviewed.

In the initial study[3] two species of mosquitoes, Aedes triseriatus and Aedes aegypti, were employed to study infection and transmission of attenuated VEE by mosquitoes. A. triseriatus was selected because of its high degree of susceptibility to infection with the virulent Trinidad strain of VEE virus as well as its capacity to transmit this agent.

Two techniques for feeding of the mosquitoes were employed: (1) direct feeding on guinea pigs infected with attenuated virus and (2) feeding by "hanging drop" utilizing a suspension containing the virus, and guinea pig erythrocytes in 1 M sucrose.

In attempts to infect A. triseriatus on guinea pigs inoculated with the attenuated virus, none of the mosquitos became infected. Mosquitoes were tested immediately after feeding and after a period of incubation. The guinea pigs employed had viremia levels of 1.7 logs. Subsequent studies showed the peak viremia obtained in all guinea pigs infected with the attenuated virus to be less than 10^2 guinea pig intraperitoneal immunizing dose$_{50}$ (GPIPID$_{50}$)/ml of blood regardless of the quantity of virus inoculated. Because the observed levels of viremia were significantly below the threshold value for Trinidad strain VEE virus in A. triseriatus this method was abandoned and the "hanging drop" technique employed.

Attempts to infect A. triseriatus by the "hanging drop" method utilized a 1 M surcrose solution containing normal guinea pig erythrocytes and attenuated virus. The virus titer of this suspension was 6.8 logs. Results are presented in Table I.

* U. S. Army Medical Unit.

TABLE I. INFECTION AND TRANSMISSION OF ATTENUATED VEE VIRUS[a] BY A. TRISERIATUS

DAYS AFTER FEEDING	NO. INFECTED/TOTAL	NO. TRANSMITTING/NO. INFECTED
0	5/5	ND[b]
5	3/5	ND
10	2/5	ND
15	2/5	ND
20	10/35	1/10[c]
35	4/20	1/4[c]

a. Each mosquito ingested approximately 1 x $10^{4.3}$ $GPIPID_{50}$ of attenuated VEE virus.
b. ND - not done.
c. Virus was demonstrated in mosquito transmitting.

Immediately and on days 5, 10, and 15 after feeding each of 5 mosquitoes was triturated and the supernatant fluid inoculated intraperitoneally (IP) into a guinea pig. Twenty and 35 days after feeding, 35 and 20 mosquitoes, respectively were individually fed on guinea pigs and then tested for virus in the same manner as with the earlier specimens. Fourteen days after inoculation or exposure all animals were challenged with approximately 1,000 guinea pig intraperitoneal lethal $dose_{50}$ ($GPIPLD_{50}$) Trinidad strain VEE virus.

Evidence that infection of the mosquitoes was accomplished is provided by the results in the center column. The day-0 values reflect infection of virus with subsequent results indicating establishment of infection; 30% of mosquitoes were infected.

Of the 35 mosquitoes employed on day 20 for transmission attempts, 10 were shown to be infected; only 1 of these transmitted virus. On day 35, 4 of 20 mosquitoes were found to be infected with 1 transmitting. The transmission rates of 1 of 10 and 1 of 4 on days 20 and 35, respectively, are considered true values in that all probes and feedings were confirmed for each mosquito.

In the second study (Table II) both A. triseriatus and A. aegypti were used. In this study the infection rate for the former was 52% and for the latter an estimated 18%. The latter value was calculated for the day 20 results. This low level of infection coupled with the number tested at each interval may in part account for the failure to demonstrate virus on days 5, 10 and 15.

As may be seen, 2 of 15 infected A. triseriatus and 2 of 6 infected A. aegypti transmitted virus.

TABLE II. INFECTION AND TRANSMISSION OF ATTENUATED VEE VIRUS[a] BY A. TRISERIATUS AND A. AEGYPTI (ROCKEFELLER STRAIN)

DAYS AFTER FEEDING	NO. INFECTED/TOTAL		NO. TRANSMITTING/NO. INFECTED	
	A. triseriatus	A. aegypti	A. triseriatus	A. aegypti
0	5/5	5/5	ND[b]	ND
5	2/5	0/5	ND	ND
10	4/5	0/5	ND	ND
15	2/5	0/5	ND	ND
20	15/29	6/33	2/15[c]	2/6

a. Each mosquito ingested approximately 1 x $10^{4.3}$ $GPIPID_{50}$ of attenuated VEE virus.
b. ND - not done.
c. Virus was demonstrated in mosquitoes transmitting.

It should be noted at this point that none of the animals inoculated with mosquito materials or which were infected following mosquito feeding exhibited any overt signs of illness. Based on these limited observations there was no evidence of a change in virulence of the attenuated virus as the result of passage in the mosquito.

The observed infection and transmission rates are low in comparison to values obtained by Corristan employing A. triseriatus and the Trinidad strain of VEE virus.[4] Under conditions comparable to those employed in these studies with attenuated virus, including concentrations of virus, Corristan found 100% became infected with 95% transmitting.

A more direct approach to the question of transmission of the virus by mosquitoes was employed in a study[5] conducted at the Communicable Disease Center by Dr. Telford Work and his staff.

Twelve laboratory personnel scheduled for immunization with the attenuated VEE vaccine generously volunteered for this study. Each person was administered 0.5 ml of vaccine which contained approximately 10^3 $GPIPID_{50}$ of virus.

Beginning 2 hr postinoculation and at 24-hr intervals thereafter for 13 days, 35-40 A. triseriatus were allowed to feed on each subject. Feedings were carried out immediately following collection of a blood sample. The mosquitoes were maintained at a temperature of 80 F and humidity of 75% for 18-20 days. During this period the daily blood samples as undiluted and as a 1:10 dilution of serum were assayed in suckling mice. The results of these assays were utilized as the basis for selecting the groups of mosquitoes which were most likely to be infected.

Mosquitoes which had fed during periods of apparent viremia were permitted to feed on guinea pigs and were then frozen for subsequent testing for presence of virus. The guinea pigs were held for 30 days at which time they were bled for serum and then challenged via the IP route with $10^{3.2}$ median mouse intracerebral lethal doses (MICLD50) of Trinidad strain VEE virus.

A total of 611 mosquitoes were assayed in suckling mice and 344 fed on guinea pigs. Although the mosquitoes fed on the subjects during periods of apparent viremia, none transmitted virus, and more importantly, none were found to be infected.

The results indicated that vaccinees did not serve as a source of virus for mosquitoes. However, it was deemed desirable to perform quantitative viremia assay in order to obtain a better estimate of the potential for vaccinees to serve as a virus source.

In the Medical Unit 2 study groups of 20 volunteers each were established. Both groups were managed in the same manner with the exception that inoculation of vaccine was accomplished with one group at 0800 hr and with the second group at 2000 hr. The reason for the difference in time of inoculation was to permit study of the effect of time of inoculation on changes in blood amino acids. As will be shown later this difference in inoculation time had no effect on incidence, magnitude or duration of viremia. The effect on blood amino acid changes is presented in the next paper.

Following collection of appropriate preinoculation serum specimens each volunteer was administered 0.5 ml of vaccine containing approximately 5,000 $GPIPID_{50}$ of virus via the subcutaneous route.

Beginning 12 hr after inoculation and every 12 hr thereafter for a total of 7 to 9 days a heparinized blood sample was obtained from each volunteer. One ml of the sample was diluted 1:10 in Hank's balanced salt solution and the remainder was placed in a sterile vial. Both samples were placed in a freezer at -65 C within 30 min of collection. Additional samples were collected as deemed appropriate by the attending physician. Results of serologic studies showed that all 40 persons were infected as evidenced by a significant increase in hemagglutination-inhibiting antibodies.

The diluted sample was employed for qualitative assay. It was planned that quantitative assays would be performed on all samples in which virus was demonstrable in the 1:10 dilution. The procedure of diluting the sample prior to freezing limited each sample to a single freeze-thaw cycle.

The qualitative assays were performed as follows. The sample was rapidly thawed and each of 4 guinea pigs weighing 250-350 gm was

inoculated IP with 2.0 ml of the sample. Fourteen days after inoculation each animal was challenged with approximately 1,000 $GPIPLD_{50}$ of Trinidad strain VEE virus. All survivors were rechallenged in a like manner following a 14-day observation period. The double challenge procedure was employed since other studies in our laboratory have shown that an occasional normal guinea pig will survive challenge. No explanation for this is apparent.

Viremia was demonstrated in 13 of the 40 volunteers. Six of these were from the group inoculated at 0800 hr with 7 in the 2000 hr group. In 8 of the 13 persons virus was demonstrated in only one sample; in one person in 2 samples and in the remaining 4 in 3, 4, 5 and 6 samples.

Quantitative assay were performed as follows. The whole blood sample was rapidly thawed and serial 10-fold dilutions made beginning at 10^{-1} through 10^{-3}. Each of 4 guinea pigs was inoculated IP with 2.0 ml of the appropriate dilution. Fourteen days after inoculation each animal was challenged with approximately 1,000 $GPIPLD_{50}$ of Trinidad strain VEE virus. As in the qualitative assay all survivors were rechallenged. After completing several assays it appeared that the maximum dilution of 10^{-3} was adequate. However, as will be seen in the results this was marginal for 1 sample.

The results of the quantitative assays performed with samples obtained from the 13 persons with demonstrable viremia are presented in Table III. The viremia levels are expressed as $\log_{10}$ of virus/ml of blood.

Two vaccinees were found to have viremia levels in excess of 2 logs. WRF had a maximum level of 2.2 logs in the sample collected at 2000 hr on day 4. The samples preceding and following this time each contained 0.7 logs of virus. REC developed a viremia of $\geq$ 3 logs at 0800 hr on day 7. It was not possible to determine the exact level since the highest dilution tested was 10^{-3}. The results indicate that the level would not be greater than 4.0 logs since 1 animal survived at the 10^{-3} dilution. The samples preceding and following this time contained 1.0 and 1.4 logs of virus, respectively.

As may be seen all other viremia levels were low. Of interest, however, is DCS who developed a viremia level of 1.2 logs on day 12 postinoculation. He had been released from isolation on day 10 and approximately 48 hr later developed symptoms which prompted his return to the hospital. Virus was not demonstrated in samples collected at 0800 and 2000 hr on days 13 and 14 and at 0800 hr on day 15.

The results of the viremia study show that while viremia does occur in persons inoculated with the attenuated VEE virus it is of low level. These low levels are significantly below the threshold of 4.5 logs[5] necessary to infect A. triseriatus with the Trinidad strain of VEE virus. The threshold is defined as the concentration of virus which will infect 5% of the test vectors.

TABLE III. VIREMIA LEVELS IN VEE VACCINEES

VACCINEE	VIREMIA (LOG_{10}) BY DAY INOCULATION													
	2	3	4		5		6		7		8		9	12
	2000	2000	0800	2000	0800	2000	0800	2000	0800	2000	0800	2000	0800	2000
WRF		1.2	0.7	2.2	0.7	≤0.3		≤0.3						
DPG		0.3		0.3		≤0.3	0.4	≤0.3	≤0.3					
REC								1.0	≥3.0	1.4	0.3			
FHM	≤0.3											≤0.3	≤0.3	
WLL									≤0.3			≤0.3		
RCW									≤0.3					
RH									0.3					
DLG											0.3			
DWR								0.3						
JDW				≤0.3										
HGG								0.3						
RR				≤0.3										
DCS														1.2

Further evidence which indicates that the viremia levels found are inadequate to infect A. triseriatus was obtained in the study at the Communicable Disease Center. Two subjects developed viremia of ≥ 2.7 logs in one case and > 3.0 logs in the other. In neither case were A. triseriatus infected when fed on these persons.

SUMMARY

The data from the artificial feeding study, those from the direct feeding study and those from the viremia study indicate that the potential for mosquitoes to be infected by feeding on persons vaccinated with the attenuated VEE virus is extremely limited if indeed nonexistent. If any potential exists it is for only very limited periods as indicated by the peak levels of viremia found in the 2 vaccinees. Thus, it is proposed that the restriction against use of the vaccine during periods when mosquitoes are present in the environment be lifted.

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CHANGES IN WHOLE BLOOD AMINO ACIDS DURING INFECTION

Ralph D. Feigin, Captain, MC*

The importance of amino acids in nutrition and their role in the intermediary metabolism of biological systems has been studied extensively. In contrast, few studies have been performed to evaluate amino acid changes in terms of the host's response to infection and/or intoxication. The first report of whole blood amino acid changes during infection in adults appeared in 1940 when Farr and his colleagues[1] reported a fall in plasma amino acid nitrogen during pneumococcal pneumonia. The only other study appeared 3 years later when Emerson et al[2] observed that similar changes did not occur with primary atypical pneumonia.

The results are presented of carefully controlled studies illustrating changes in whole blood amino acid of volunteers infected with respiratory acquired tularemia and following immunization with live attenuated Venezuelan equine encephalomyelitis (VEE) vaccine. Blood amino acid changes in mice following staphylococcal enterotoxin B (SEB) administration are also presented.

The work to be reported here has employed the chromatographic technique of Efron and her co-workers,[3] permitting the analysis of amino acids on 6 µL of whole blood. The procedure was modified as follows: Ninhydrin stained chromatograms were dried and developed in a chromatography oven at 110 C for 8 min. Each chromatogram was quantitated by densitometric analysis employing an Analytrol RB densitometer to obtain an integrated tracing.

Figure 1 shows a typical result following chromatography and densitometry in a CD-1, 12-gm male mouse. The integrated value of each amino acid detectable as a single spot was converted to µg/0.006 ml by comparison to a standard curve. Standard curves were prepared by analysis of increasing concentrations of each amino acid in a similar manner. The summation of the integrated values of each singly identified amino acid, as well as values obtained for amino acid groups, gave a total value that has been equated with the term total amino acids. The technique permits concurrent analysis of as many as 60 samples per chromatography tank minimizing changes attributable to methodological variables.

During the course of these investigations it became apparent that whole blood amino acids in man are not maintained at a constant level throughout the day but that a pattern exists such that levels at 2000 hr are consistently higher than those obtained at 0800 hr $p < 0.05$. These conclusions have been reached on the basis of amino acid analysis of blood obtained from 56 men at 0800, 1400 and 2000 hr on one day and at 0800 hr the following day. In addition, specimens from 7 men have been obtained at the same time intervals for 8 consecutive days and from 2 men for 21 consecutive days. These findings have been confirmed by performing alpha amino nitrogen determinations according

FIGURE I. TOTAL AMINO ACIDS IN A CD-I, 12 gm, MALE MOUSE.

to the method of Moore and Stein[4] as well as by amino acid analysis performed by Dr. Mary Efron of the Massachusetts General Hospital, Boston, Massachusetts, utilizing a Technicon amino acid analyzer. Thus, to interpret amino acid changes during the course of infection, cognizance must be taken of those changes which can be attributed to amino acid periodicity.

The effects of respiratory-acquired Pasteurella tularensis infection upon whole blood amino acids has been studied in volunteers. Sixteen men were exposed to an average of 25,000 viable SCHU-S4 cells. Two individuals were sham-exposed but not infected. On the day prior to exposure, blood was obtained by venipuncture from each individual at 0800, 1400 and 2000 hr. Thereafter, blood was obtained at 0800 and 2000 hr daily. Rectal temperatures were taken every 6 hr from the time of admission until the time of discharge. Following exposure, signs and symptoms of each subject were evaluated and illness categorized as mild or severe.

Figure 2 illustrates the composite amino acid responses of the 16 infected subjects. The solid black line is the mean ± 1 SE of the total integrated value of severe cases while the solid white line is that of the milder cases. Day 0 is day of exposure. Within 12-60 hr of exposure, a proportional decrease in each amino acid ($p < 0.01$) occurred in all 16 subjects regardless of severity of illness. This decrease preceded the onset of fever or any other clinical symptomatology by 12-36 hr in every subject. The decrease became maximal 2.28 days postexposure when the mean ± 1 SE decrease of 43 ± 4% was noted. The range was 18-63%. The first signs of clinical illness were noted on day 2 in 5 subjects, on day 3 in 10 subjects, and on day 4 in one subject. The decreases in amino acid concentrations were greater in those patients with the more severe clinical illnesses.

Within 6-72 hr of the onset of fever, an increase in total amino acid concentration above normal was noted. This increase is only present in those subjects with the more severe clinical illnesses.

Typical responses of an infected individual are shown in Figure 3. Rectal temperatures are shown above. The lower line presents total amino acids. Day 0, day of exposure, is indicated by the arrow. Tetracycline therapy was started on day 5. A marked drop in total amino acids is evident 24 hr after exposure and 48 hr prior to onset of fever. A secondary rise in total amino acids is seen 72 hr following onset of fever, with subsequent return to the normal range.

Whole blood amino acids of the 2 noninfected subjects remained relatively constant throughout the observation period. The responses of one of these subjects is illustrated in Figure 4. Each control was given a course of tetracycline to evaluate its effect if any upon whole blood amino acids. As can be seen, none was noted. The diurnal rhythmicity of whole blood amino acids in man is well demonstrated here. The 0800-hr specimens are consistently lower than the 2000-hr specimens. A striking correlation is noted between normal temperature periodicity and corresponding amino acid patterns but a causal relationship cannot be implied.

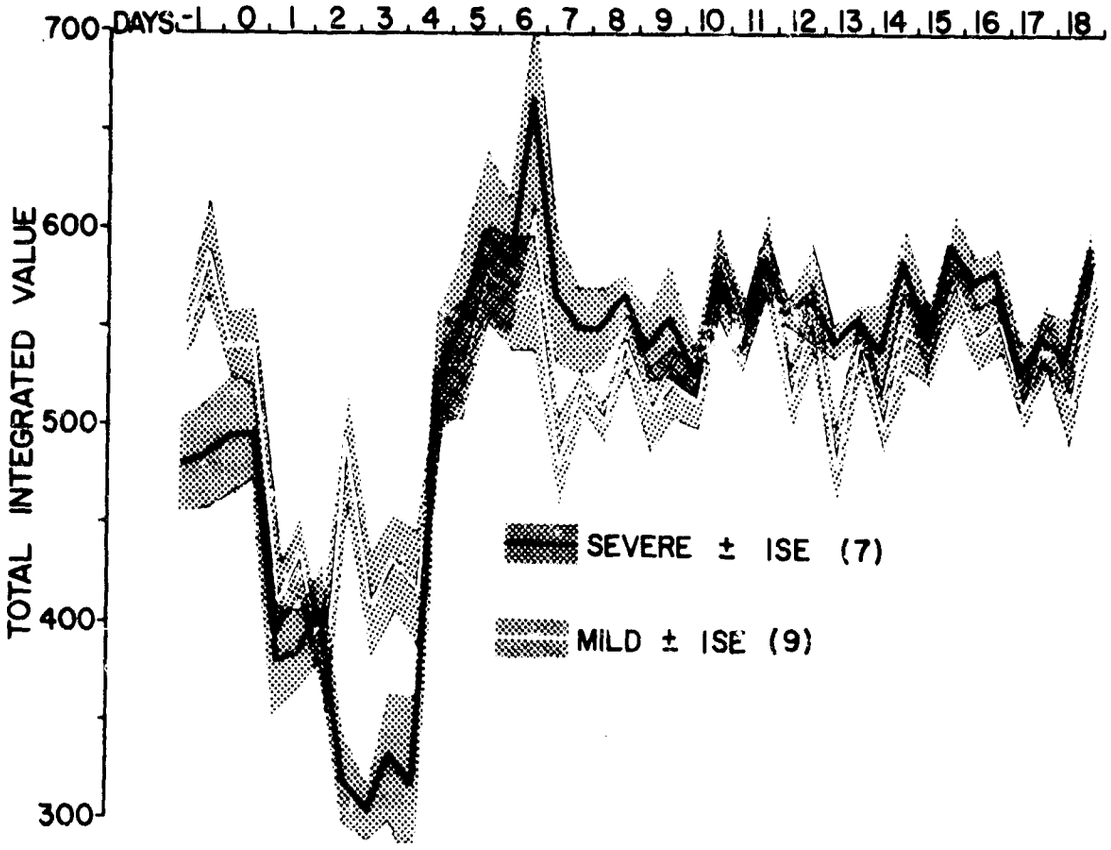

FIGURE 2. TOTAL AMINO ACID RESPONSE IN RESPIRATORY TULAREMIA.

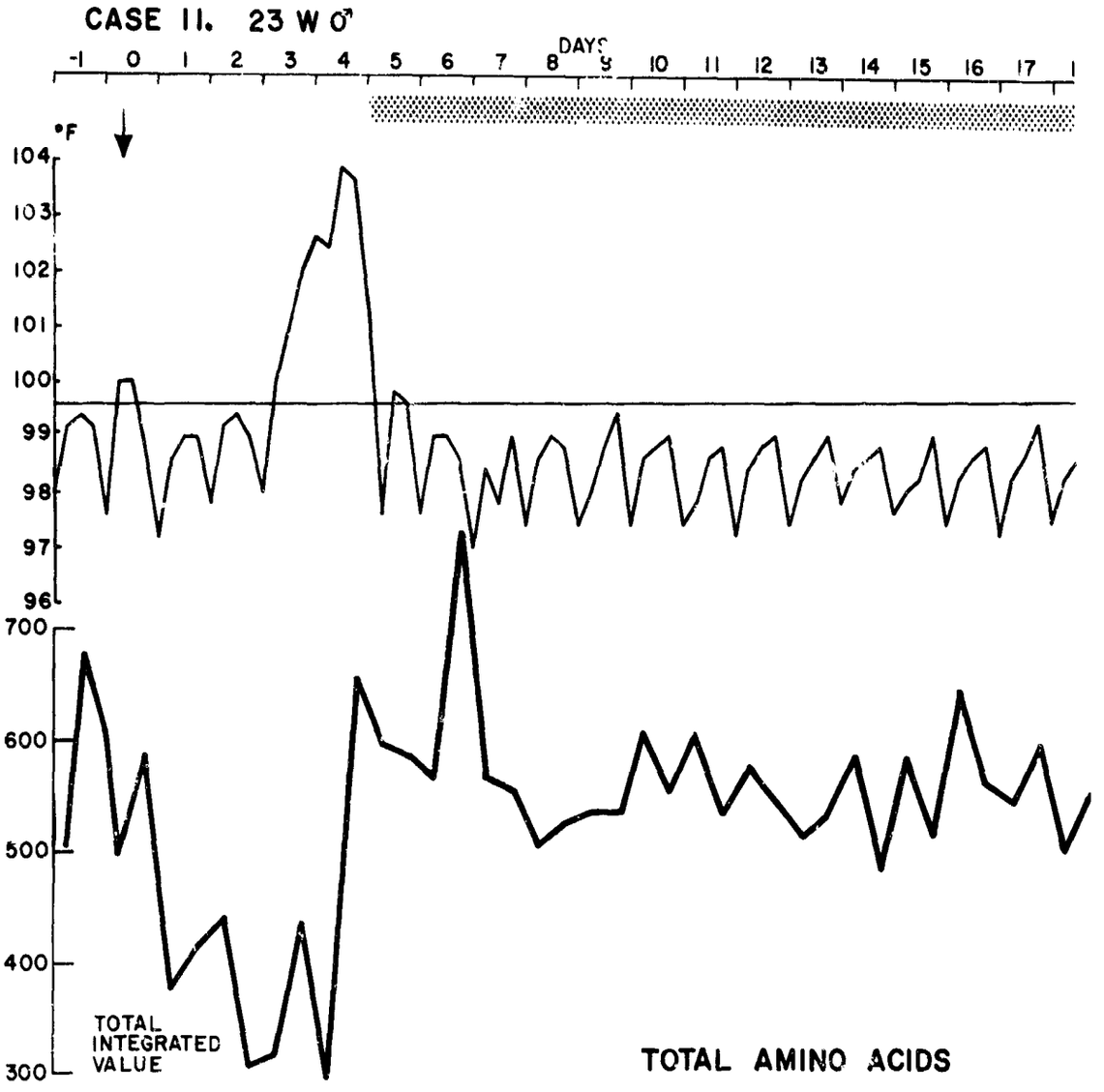

FIGURE 3. TEMPERATURE AND TOTAL AMINO ACID RESPONSES IN RESPIRATORY TULAREMIA (CASE II). TETRACYCLINE THERAPY SHOWN AT TOP. * AT 0800 HR SAMPLING TIME.

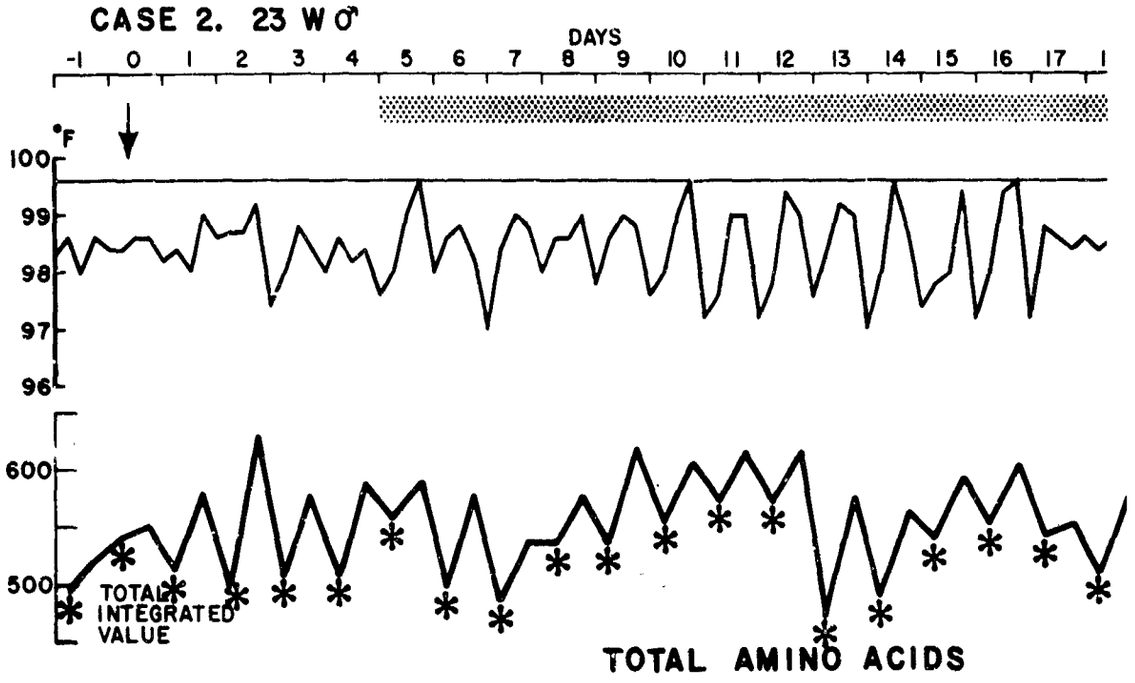

FIGURE 4. TEMPERATURE AND TOTAL AMINO ACID RESPONSES IN SHAM-EXPOSED INDIVIDUAL (CASE 2). TETRACYCLINE THERAPY SHOWN AT TOP.

The factors reponsible for the decrease in amino acid concentration are not readily apparent. Beisel's work[5] as well as our own revealed a diminished urinary excretion of amino acids during the period of time that blood amino acid concentrations were lowest, therefore, low blood level could not be accounted for by urinary losses. Woodward et al[6] observed that cystine, an essential metabolite for P. tularensis, disappeared from the blood of rats infected with highly virulent strains of the organism. He also noted lesser decreases in the concentrations of other amino acids. In contrast, killed or living avirulent organisms did not induce similar changes. Woodward hypothesized that localization of this organism within the liver and spleen might result in utilization of free amino acids directly from the blood as it filtered through these organs. Whether the reduction in whole blood amino acids in man infected with tularemia could be explained by this hypothesis is speculative. However, our findings of a proportional rather than selective decrease in cystine suggest that other factors might be operative.

Significant increases in beta and gamma globulins as well as associated decreases in blood urea nitrogen, nonprotein nitrogen, and serum albumin have been described by Sbarra et al[7] within 24 hr of tularemia infection in rats. Cognizant of these early changes in animals, it is attractive to speculate that P. tularensis in man has initiated similar metabolic changes resulting in utilization of whole blood amino acids for new protein synthesis. Confirmation of this hypothesis will require additional study.

Increased excretion of glucocorticoids during human tularemia infection was demonstrated by Beisel[5] to occur with the onset of fever and to abate concomitantly with clinical improvement. Furthermore, during infection, adrenal over activity was roughly proportional to the clinical severity of the disease process. The catabolic effect of glucocorticoids on body protein is well known. Amino acid increases were seen following fever in our subjects with the more severe clinical illnesses. This rise in amino acids following fever might be the result of a steroid catabolic effect.

A second study was undertaken to evaluate what effect, if any, a viral infection might have upon blood amino acids.

The existence of diurnal periodicity of whole blood amino acid concentration coupled with observations in the literature that animals exposed to the same dose of a toxin at different times during the day showed widely different mortality rates, prompted us to study the effect of immunization upon blood amino acids in volunteers at 2 time periods. This was incorporated into the study described in the previous two papers.

The composite amino acid responses are shown in Figure 5. Determinations at 0800 hr are designated by asterisks.

Ten in the 0800-hr group developed fever > 100 F (rectally). In all 20 the normal amino acid diurnal periodicity was affected as seen in the curve

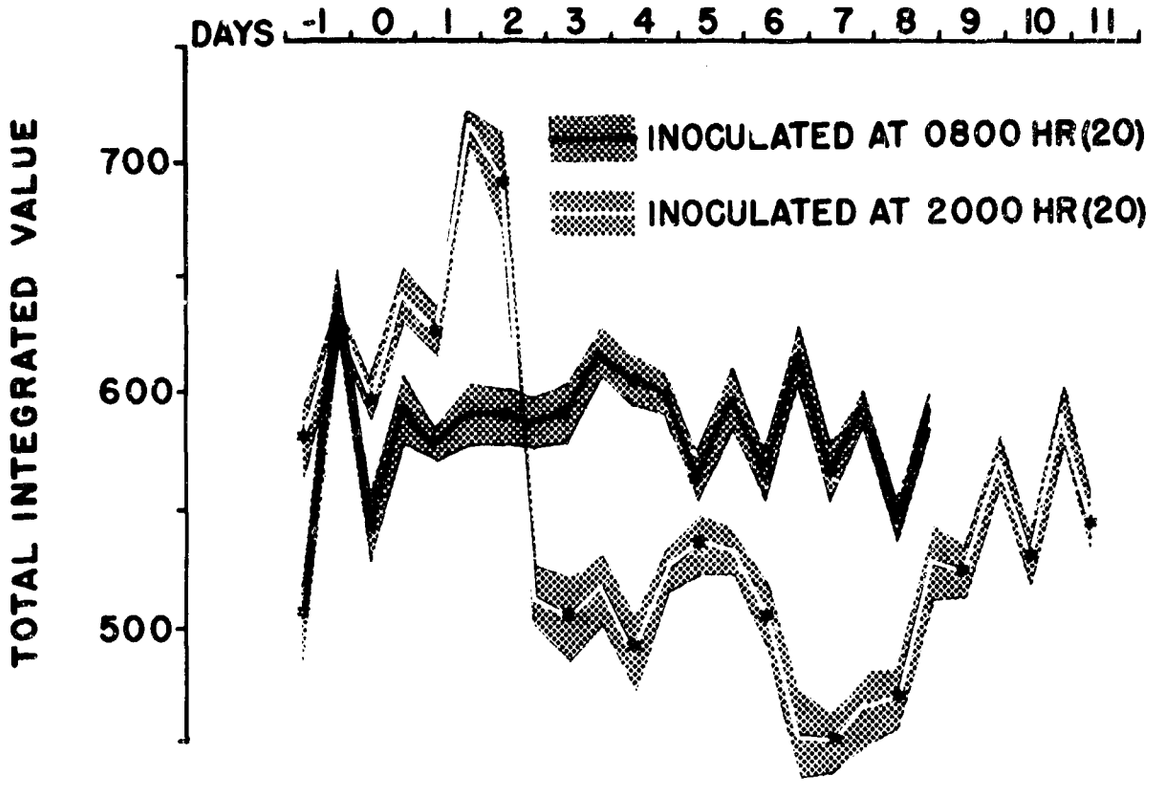

FIGURE 5. TOTAL AMINO ACID RESPONSE TO VEE IMMUNIZATION AT 0800 AND 2000 HR. * SHOWS 0800 HR. SAMPLING TIMES FOR 2000-HR GROUP.

with the solid black line. Note the normal rhythmicity preinoculation, with loss of rhythm on day 1, reversal on day 2, normal periodicity on day 3 and a second reversal of rhythm on day 4 prior to resumption of the normal periodicity.

The curve with the white line shows the composite results of the 20 men inoculated at 2000 hr. Eleven of these subjects developed fever or other symptomatology at some time during the observation period. All 20 volunteers developed a reversal of their circadian periodicity lasting a minimum of 2 days to as long as 8 days. In addition, all manifested elevated amino acid levels on day 1 and 2 postimmunization and depressed amino acid levels on days 3-7. The presence of proline was detected in many specimens for the first time, its normal concentration being at or below the levels detectable by this system of analysis even when specific isatin stains are utilized.

Serum specimens obtained from the 2 individuals whose amino acid changes represented the minimal and maximal serum proline increases as determined in our system, were subjected to precise quantitation in a Technicon amino acid analyzer. Large increases in proline and glutamic acid with lesser increases in citrulline, ornithine and arginine were found. In addition, a striking decrease in glutamine was apparent.

Figure 6 shows the results from the patient with the smallest increase in blood proline. The metabolic pathway is summarized on the bottom of the figure. Proline is metabolized to glutamic acid which in the presence of glutamine synthetase goes to glutamine. A 7-fold increase in proline concentration is seen from the very low pretest values. Similarly a 4-fold increase in glutamic acid is apparent. In contrast, glutamine decreased to 50% of its pretest concentration.

Figure 7 shows the results in the subject with maximal proline changes. An 83-fold proline increase is noted with a subsequent fall to pretest values. This increase is in the order of magnitude of that seen in children with hyperprolinemia. Similarly, glutamic acid increased 17-fold. In this case, glutamine decreased to 10% of its pretest value. Because both proline and glutamic acid increases were seen we cannot postulate a block similar to that which has been described in either type of hyperprolinemia. Rather, we feel that a block or inhibition of glutamine synthetase could account for the metabolic changes noted. Such a block has not to our knowledge been previously demonstrated in any inborn error of amino acid metabolism. The blockage of glutamine synthetase may also account for the smaller but still significant increases seen in ornithine, arginine and citrulline because of the intimate relationship of the proline and urea cycles.

The more pronounced changes manifested by the group inoculated at 2000 hr deserves comment. Halberg[8] showed that susceptibility of mice to Escherichia coli lipopolysacharide injected intraperitoneally (IP) as measured by death differed with the time of inoculation. The death rate of mice injected at 1600 hr was 80% greater than that observed at 2400 hr

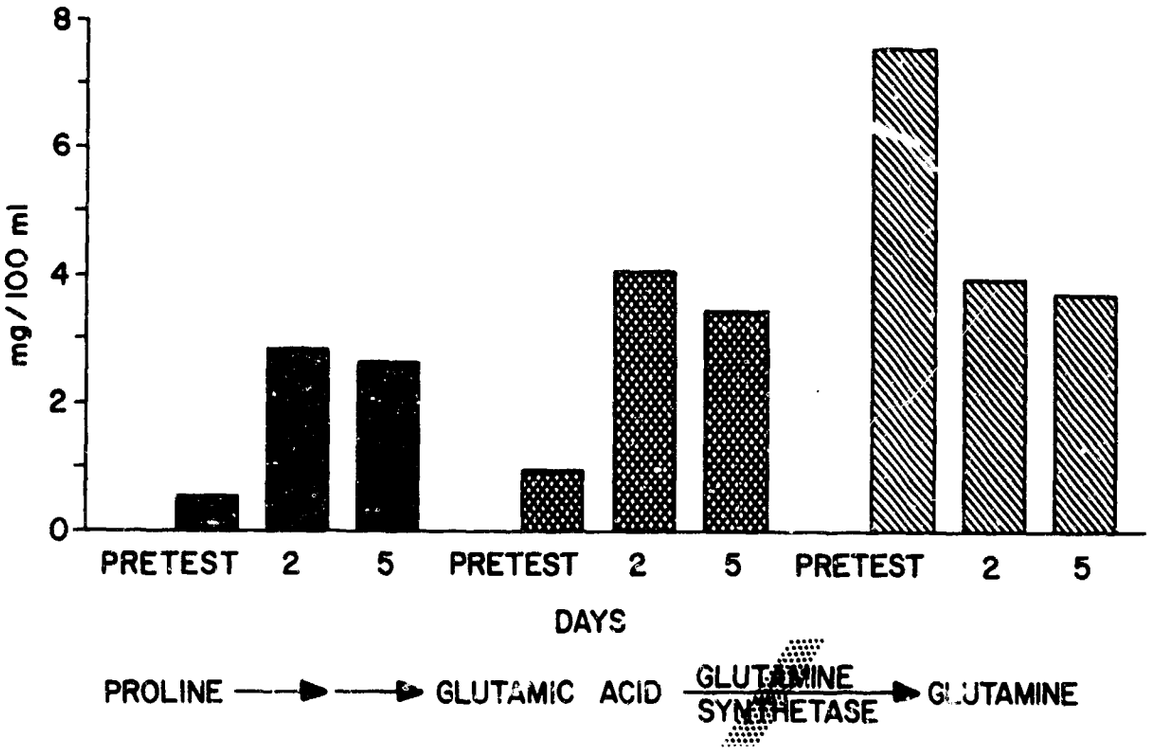

FIGURE 6. CHANGES IN SELECTED AMINO ACIDS FOLLOWING VEE IMMUNIZATION AT 2000 HR (CASE I). METABOLIC PATHWAY SHOWN BELOW.

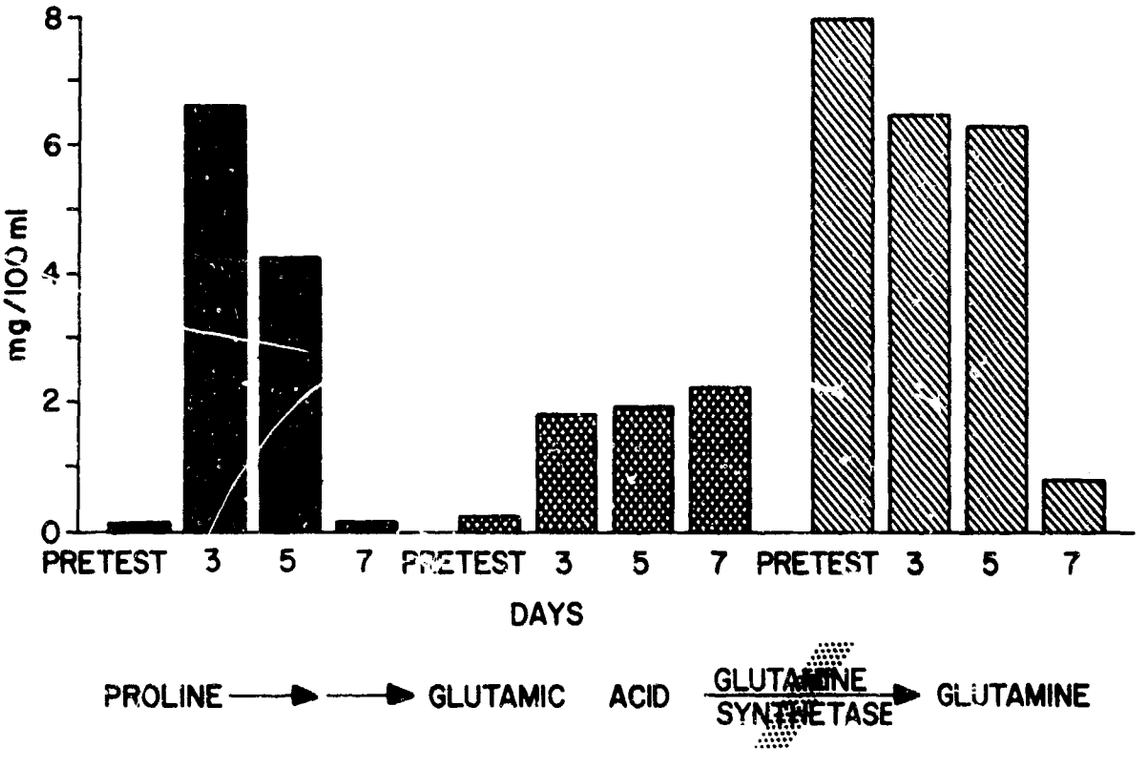

FIGURE 7. CHANGES IN SELECTED AMINO ACID FOLLOWING VEE IMMUNIZATION AT 2000 HR (CASE 2). METABOLIC PATHWAY SHOWN BELOW.

when the same dose was utilized. In a similar way, it is apparent that infection of man with VEE at 2000 hr results in biochemical changes which are more pronounced than in men inoculated at 0800 hr.

In a third study, a highly purified preparation of SEB Lot 14-30 supplied by Dr. Schantz and his associates has been studied in 10-14 gm CD-1 (Charles River) mice. Early in the course of this study, a significant difference in the levels of whole blood amino acids was noted in mice in samples taken at different times of the day (solid line, Figure 8).

Each point represents the mean ± 1 SE of the total integrated value for 25 separate determinations. The shaded area represents the period of total darkness. It should be noted that the intermouse variability at a given point is markedly less than variation due to circadian periodicity.

A similar rhythmicity with respect to adrenocorticosteroids is well known. To evaluate a possible relationship, adrenalectomized mice of the same strain and weight were employed in a similar study; the results are shown by the dashed line. It can be seen that adrenalectomy has caused phase shift but not abolished circadian rhythmicity.

With this variation in mind, an LD_{20} of SEB was given IP to 40 mice at 0800 hr. One-half of the group was sacrificed at 4 hr and the other at 24 hr postchallenge. Following SEB challenge, all whole blood amino acid levels appeared to be unchanged except for tryptophan. Therefore, these specimens were rerun, for more precise quantitation of tryptophan by the modified Fischl procedure.[9] To interpret these changes, all samples from controls shown on the previous figure were analyzed in a similar manner. The circadian periodicity of tryptophan is shown in Figure 9 and reported in mg/100 ml.

The mean ± 1 SE are plotted. The results in blood obtained at 4 and 24 hr postchallenge with SEB are shown by the dashed line. The importance of understanding circadian periodicity is clear. Without this information, it could be assumed that tryptophan levels were decreased at 4 hr whereas they were increased when compared to control samples (solid line) taken at the same time. A further increase is apparent 24 hr postchallenge.

Since blood tryptophan is known to be influenced by adrenal steroids, studies were performed in mice to whom cortisol was given as well as in adrenalectomized mice. Cortisol, 0.75 mg, was given IP to 40 normal mice; 20 each were sacrificed at comparable intervals. The line of dashes and dots reveals an increase in tryptophan above corresponding control values with a return to normal at 24 hr.

Whole blood tryptophan levels in adrenalectomized mice are illustrated in Figure 10. It should be noted that the circadian rhythm has been reversed. The dashed line shows that an LD_{20} of SEB given adrenalectomized mice has no effect on the tryptophan level. In contrast, the dash-dot line

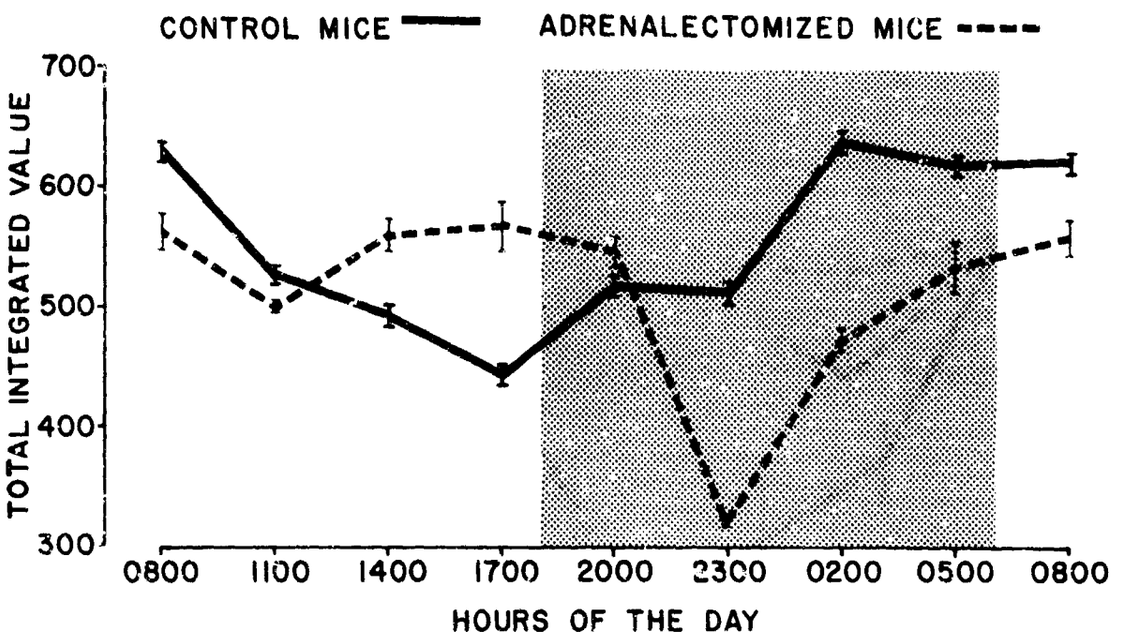

FIGURE 6. TOTAL AMINO ACIDS IN ADRENALECTOMIZED AND CONTROL, CD-1 MICE (20 / POINT).

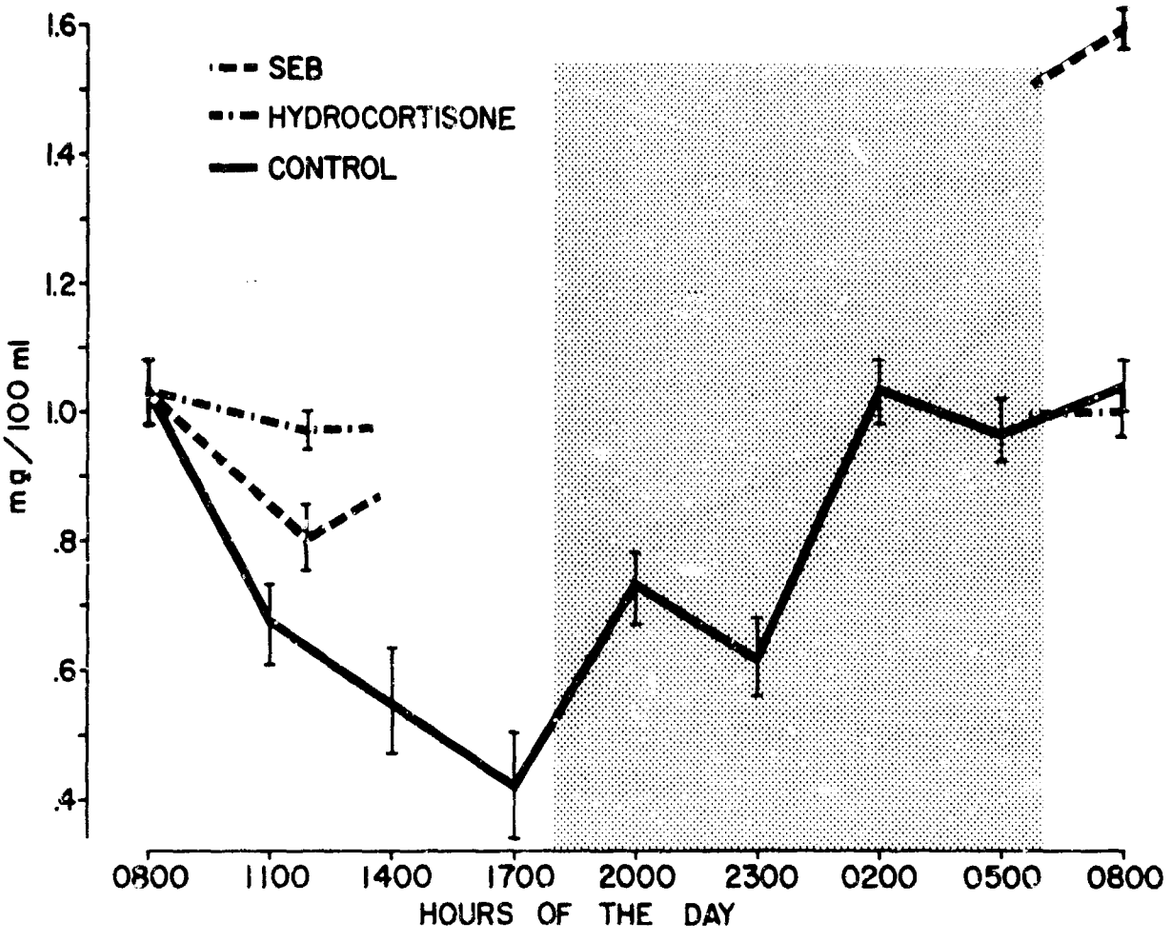

FIGURE 9. WHOLE BLOOD TRYPTOPHAN IN CD-1 MICE (20/POINT) FOLLOWING ADMINISTRATION OF SEB AND HYDROCORTISONE.

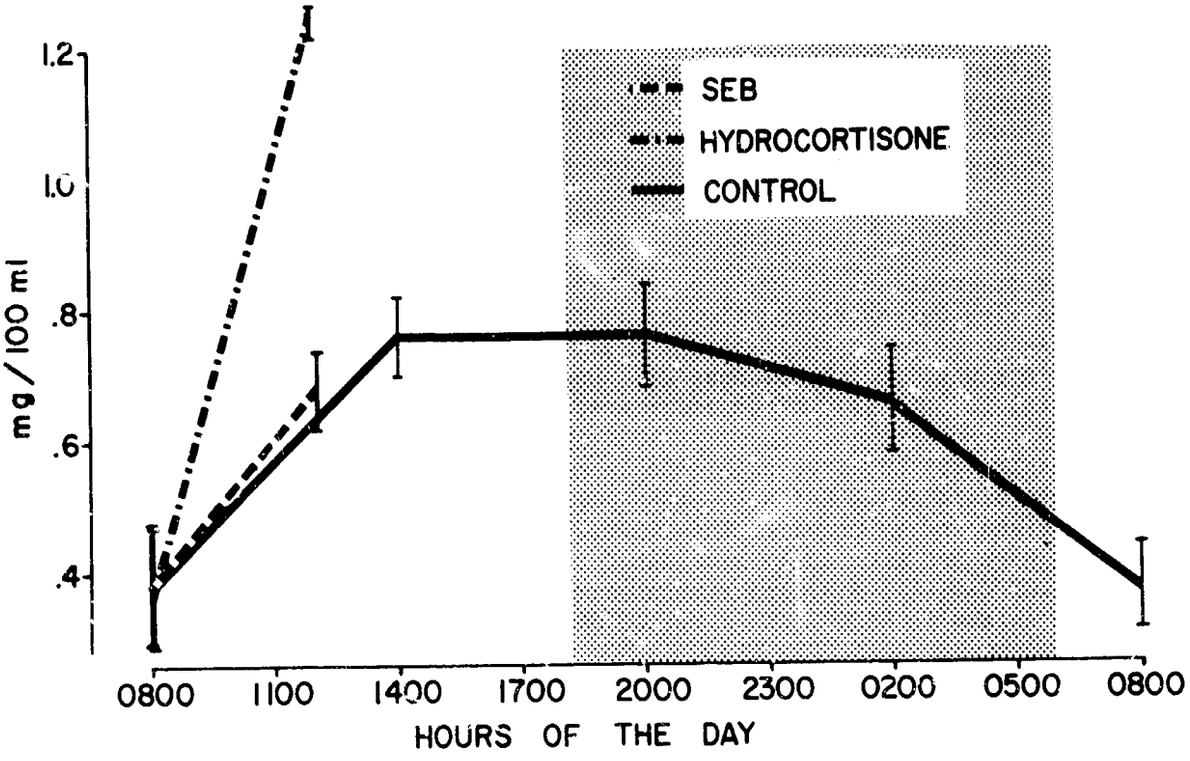

FIGURE 10. WHOLE BLOOD TRYPTOPHAN IN ADRENALECTOMIZED CD-1 MICE FOLLOWING ADMINISTRATION OF SEB AND HYDROCORTISONE

shows that 0.75 mg of cortisol results in a marked increase in circulating tryptophan. All other whole blood amino acids remained within the normal range following SEB or cortisol in both normal and adrenalectomized mice.

These findings suggest that the tryptophan rise seen following SEB is dependent upon intact adrenocortical function. SEB intoxication in the presence of endogeneous glucocorticoids may engender protein catabolism in certain tissues with liberation of free amino acids. However, since increases in other whole blood amino acids did not occur, a steroid catabolic effect cannot explain the tryptophan rise seen 4 and 24 hr after SEB administration. The liver, a known site of increased protein synthesis following cortisol administration, may utilize all the free blood amino acids except tryptophan. Bacterial endotoxins are known to inhibit hepatic tryptophan pyrrolase. Sustained elevation of whole blood tryptophan may result from a similar enzymatic inhibition. This inhibition has in fact been documented in studies of Rapoport and colleagues.[10]

SUMMARY

Our observations of whole blood amino acid periodicity in mice and men coupled with Squibb's report[11] of a circadian rhythmicity of serum and liver amino acids in chickens, strongly suggests that this phenomenon may occur in a wide variety of species. Cognizance of circadian periodicity is essential to interpret blood amino acid change in infection.

Respiratory acquired tularemia in man caused a significant decrease in whole blood amino acids 12-36 hr postexposure and prior to the onset of other signs and symptoms of infection. The factors responsible for this change have not been fully elucidated. The rise in amino acids noted following fever in patients with more severe illness has been attributed to the catabolic effect of increased quantities of circulating glucocorticoids.

Live attenuated VEE virus vaccine caused a reversal of normal diurnal periodicity of whole blood amino acids regardless of time of inoculation. The greater biochemical effect noted in the group inoculated at 2000 hr suggests that the greater or lesser effect of an infectious material upon the human host may be dependent upon the time of exposure. The unusual rise in proline is of the order of that seen in inborn errors of metabolism and may be explained by an infection related enzymatic inhibition.

SEB in CD-1 strain male mice caused an elevation of whole blood tryptophan. This selective elevation has been attributed to an inhibition of tryptophan pyrrolase.

It is apparent that these studies are at best a beginning. They have, however, confirmed that metabolic changes resulting from host:pathogen interaction occur early in infection and prior to the onset of signs and symptoms. Moreover, these changes can be detected by a practical method

utilizing small quantities of blood. The practicality of the method for the early diagnosis of infectious disease depends upon the specificity of the change for a given disease entity. This can only be determined by additional studies of amino acid changes in other infections.

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SEROLOGICAL STUDIES ON STAPHYLOCOCCAL ENTEROTOXIN B

Virginia G. McGann, Ph.D.*

Investigations on the immunologic response to staphylococcal enterotoxin B (SEB), have been confined to examination of the role of circulating antibody. Serologic methods used for detection and measurement of antibody were: (1) hemagglutination with bis-diazotized benzidine-treated erythrocytes (BDB-HA), (2) precipitation in agar gel (PPT), and (3) enterotoxin-combining activity, as detected with agar gel techniques (ECA). When we reported to the Commission last year,[1/] we had problems with poor reproducibility of HA titers and low sensitivity of precipitin and toxin-combining techniques. By certain modifications in procedure, these problems were resolved. This has made possible valid comparison of the 3 techniques for measuring antibody, investigations on the feasibility of using antibody measurement as an estimate of resistance to challenge, and studies on antibody response after exposure to enterotoxin.

The procedure for titrating toxin-combining activity was essentially the same as previously described. Increasing concentrations of toxin were incubated with constant amounts of test serum, and the mixtures were tested for excess of either component by observing their reactions with reference toxin and antitoxin in agar gel plates. If a mixture contained excess antibody, a line of precipitate formed between the mixture reservoir and the reference toxin, if excess toxin, between the mixture reservoir and reference antitoxin. The midpoint of the zone with no visible reaction was taken as a measure of toxin-combining activity, previously called equivalence value, and reported in terms of μg toxin/ml serum.

Relationship of BDB-HA, PPT and ECA. Data available at the time of the previous report indicated that there was good correlation between precipitin and combining activity but poor correlation between these measures and hemagglutinin. After modifications in technique, it became necessary to reevaluate these conclusions.

Concurrent titrations of sera from susceptible animals before and after exposure to enterotoxin indicated that HA and combining activity were almost equally sensitive methods for detecting antibody. Incidence and median titers of antibody, as measured by the three methods, in nonexperienced men and monkeys are summarized in Table I. Approximately 65% of the human and 20% of the monkey populations were antibody-positive by HA and ECA. In populations such as these, with no known prior experience, the number of individuals with precipitins was consistently lower than the number with hemagglutinins. Sera containing precipitins usually had hemagglutinin titers of $\geq$ 1:640. Median titers appeared to be higher in the monkey than in the human population, but after conditioning for 2 or 3 months, titers of positive monkeys decreased to approximately the level of the human titers.

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This suggested that the monkeys might have had a fairly recent experience with toxin or with a related antigen.

TABLE I. INCIDENCE AND MEDIAN TITER OF ANTIBODY IN NONEXPERIENCED MEN AND MONKEYS

SPECIES	NO. OF SUBJECTS	TEST	% POSITIVE	MEDIAN TITER
Man	198	BDB-HA	67	60
		PPT	28	1
		ECA	62	0.05
Monkey	333	BDB-HA	18	320
		PPT		2
		ECA		0.5

Overall inspection of individual monkey sera obtained before and after known exposure to toxin indicated that the 3 techniques were probably measuring the same or closely related antigen-antibody systems (Figure 1). Titers were converted to a $\log_{10}$ base; mean precipitin titers and mean toxin-combining activity showed a straight-line relationship with hemagglutinin titers between $\log_{10}$ values of 1 to 5 (corresponding to titers of 1:160-1:80,000). Slopes of the lines were approximately 0.9. It is evident that a straight-line relationship also exists between precipitin and combining activity. In sera with hemagglutinin titers < 1:80, precipitins could rarely be demonstrated. Equivalence values appeared to be related to history of exposure. In sera from nonexperienced animals, combining activity was lower than expected, whereas in sera from rechallenged animals the values were higher than expected. At hemagglutinin titers > 1:80,000, the range in which variability in titer increased, precipitin and combining values apparently reached a plateau.

Relationship of Circulating Antibody to Resistance. In the previous report[1] we concluded on the basis of limited data that precipitating antibody was probably more representative of protective status than hemagglutinating antibody. Later work has made it necessary to reexamine this conclusion.

Experienced monkeys that survived an initial exposure of 30-300 µg toxin/kg body weight were divided into 5 groups on the basis on HA titer 10-12 months after initial challenge. The groups were rechallenged at 12-14 months with $\log_{10}$ increments of toxin, from 0.1 to 1,000 µg toxin/kg body weight. Nonexperienced, antibody-negative monkeys constituted control groups. Sera obtained immediately prior to challenge were titrated for antibody, and prechallenge titers were compared with overt response.

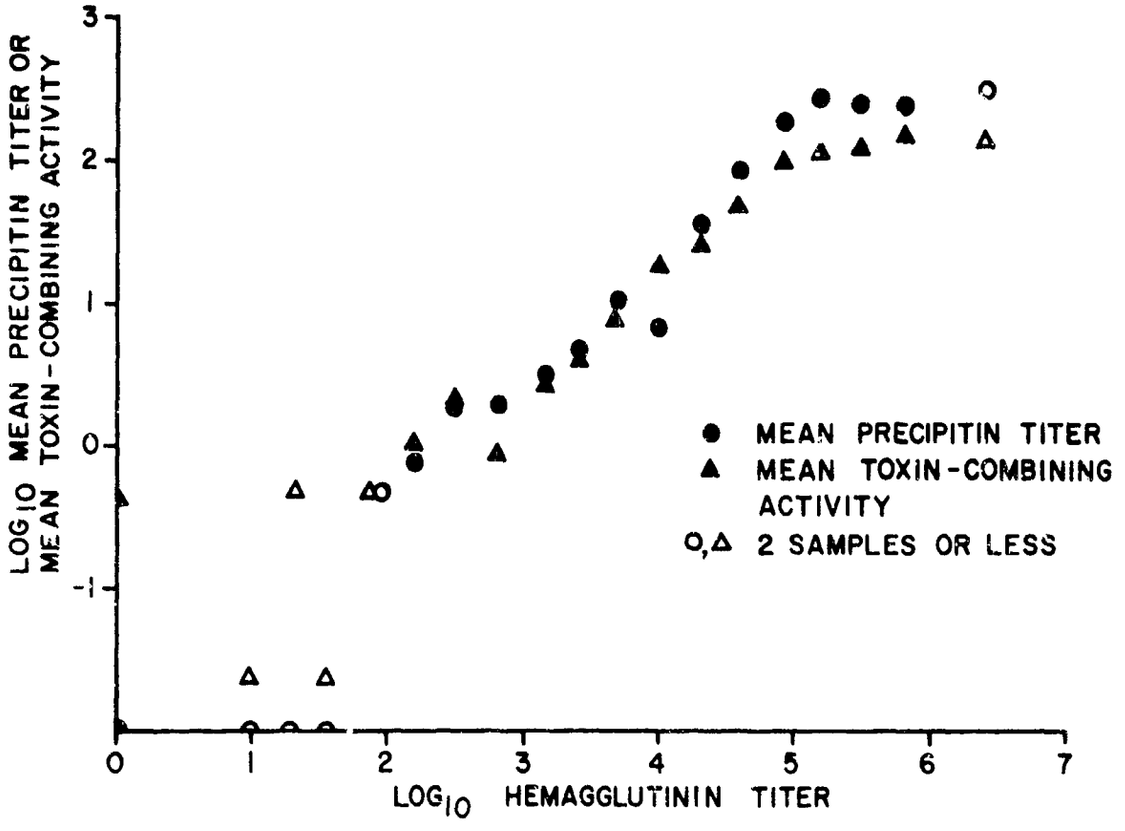

FIGURE I. RELATIONSHIP OF MEAN PRECIPITIN TITER AND MEAN TOXIN-COMBINING ACTIVITY TO HA TITER.

Table II shows the incidence of overt response to challenge of nonexperienced and experienced monkeys as related to the presence of hemagglutinating antibody in prechallenge sera. Essentially the same

TABLE II. INCIDENCE OF ILLNESS AND LETHAL RESPONSE OF NONEXPERIENCED AND EXPERIENCED MONKEYS, AS RELATED TO THE PRESENCE OF HEMAGGLUTININS IN PRECHALLENGE SERA

RESPONSE	CHALLENGE DOSE (μg/kg)	NONEXPERIENCED		EXPERIENCED			
		BDB-HA Negative		BDB-HA Negative		BDB-HA Positive	
		R/T[a]	%	R/T	%	R/T	%
Illness ≤ 12 hr	0.1- 10	16/21	76	5/11	45	4/44	9
	100-1000	12/12	100			23/32	72
Death ≤ 10 days	0.1- 10	0/21	0	1/11	9	1/44	2
	100-1000	10/12	83			3/32	10

a. Number showing overt response/Number challenged.

results were obtained if the monkeys were grouped on the basis of their prechallenge precipitating antibody. In this and other studies there was no significant difference between antibody-negative animals and animals with HA titers < 1:100; consequently, prechallenge sera were considered to be positive for hemagglutinin if the titer was > 1:100. Overt response was defined as emesis and/or diarrhea within 12 hr, or death within 10 days after challenge with doses of 0.1-10 or 100-1,000 μg/kg. None of the nonexperienced animals had detectable antibody of any kind, and significant numbers became ill at all challenge levels; 10 of 12 died after exposure to high doses.

Of the experienced animals exposed to low doses, 11 of 55 had no hemagglutinins. Although lacking demonstrable antibody, these groups showed significant resistance to illness at low doses, but less than experienced animals with antibody. Essentially all animals exposed to high doses had prechallenge antibody, and showed some resistance to illness. With regard to the lethal effect of toxin, too few deaths occurred after challenge with low doses for detection of statistically significant differences between groups. At high challenge doses, however, significant protection against death was evident in antibody-positive animals.

In Table III overt response of nonexperienced and experienced animals as related to toxin-combining antibody is presented. Results which are tabulated as relative combining power represent the ratio of combining

TABLE III. RELATIONSHIP OF RELATIVE TOXIN-COMBINING ACTIVITY OF SERUM AT TIME OF CHALLENGE TO ILLNESS AND LETHAL RESPONSE OF MONKEYS

EXPERIENCE	CHALLENGE DOSE (μg/kg)	RESPONSE	R/T[a] Relative toxin-combining activity[b]			
			0	0.001-0.9	1-10	20-100
None	0.1- 10	Illness	15/20	1/1		
		Death	0/20	0/1		
	100-1000	Illness	11/11	1/1		
		Death	9/11	1/1		
IV Challenge 12-14 months previously	0.1- 10	Illness	5/12	1/5	0/13	3/25
		Death	1/12	0/5	0/13	1/25
	100-1000	Illness		18/22	5/10	
		Death		3/22	0/10	

a. Number showing overt response/number challenged.
b. EGA/ml serum per μg circulating toxin (estimated) per ml serum.

activity (equivalence value)/ml serum to circulating toxin/ml serum at time of challenge. The estimate was made on the assumption that toxin was equally distributed throughout the circulation at challenge, although we recognize that distribution would be altered by specific affinity of toxin for host tissue or by the presence of antibody. Sera from nonexperienced animals demonstrated relative combining activities of ≤ 0.001.

Experienced animals were more resistant to illness than similar nonexperienced animals. As relative combining power increased to values of 1-10, resistance to illness also increased. The same was true of resistance to the lethal effect. At relative activities of 20-100, however, 3 of 25 animals became ill and one of the 3 died. These numbers are not statistically significant but they suggest the possibility of hypersensitivity reactions; the one death occurred within 18 hr of challenge with 1 μg toxin/kg; all other animals challenged at that level survived for at least 10 days. At autopsy, gross examination revealed nothing remarkable.

Titrations for combining power indicated that the activity of prechallenge sera from experienced animals represented at least 2 toxin-combining reactions. Antibody in one reaction was identical with the major antibody in hyperimmune reference serum; the other antibody was not detectable in the reference serum. In 75 monkeys, antibody identical with the major component of hyperimmune

serum was the only detectable antibody in 44%, the major component in 11%, and a minor component, or lacking, in 45% of the animals. Since similar results were observed in surveys of human sera, an effort was made to evaluate the relative importance of total antibody content and of the hyperimmune-like fraction.

X^2 values were used to estimate probability of relationship between prechallenge antibody and resistance. Antibody measures were tested independently. For illness at low doses and death at high doses, X^2 values based on total antibody, regardless of type, were highly significant ($p < 0.001$), indicating relationship between total antibody and resistance. For illness at high doses, the significance of X^2 values for total antibody was considerably lower ($p < 0.05$) and hyperimmune-type antibody appeared to be a better measure of resistance.

Scattered data suggest that antibody in nonexperienced monkeys has the same relationship to protection as antibody in experienced animals. Two groups of 4 nonexperienced, antibody-positive monkeys were challenged with 10 or 100 μg/kg. Relative combining power of sera from all animals challenged with the low dose was ≥ 10, and the monkeys showed no overt response. One of 4 monkeys challenged with the higher dose survived. Relative combining activity of prechallenge serum of the survivor was 0.01, and of the 3 that died, < 0.001. Moreover, in skin-test experiments with nonexperienced monkeys, antibody-negative animals became ill, while antibody-positive responded like experienced, positive animals.

Antibody Response to Enterotoxin. In previous studies most monkeys that had no prechallenge antibody showed no antibody response to toxin doses of 30 to 300 μg/kg within the standard observation period of 10 to 14 days, suggesting that purified toxin might have low antigenicity, or that antibody response might be delayed. In the past year serial bleedings were obtained from groups of antitoxin-treated monkeys and from corresponding untreated controls that survived doses of 30 to 300 μg/kg.

In Figure 2 incidence of antibody response in monkeys treated at onset of illness with monkey, human or horse antitoxin is compared with the incidence of response in untreated surviving control animals. All animals under consideration had no detectable prechallenge antibody. Sera obtained at various times after challenge were tested for hemagglutinins and for precipitins. There were 10 survivors in the untreated control group, 13 in a group treated with alpha-globulin from human convalescent serum, 32 in a group treated with pepsin-digested globulins from hyperimmune horse serum, and 23 in a group treated with convalescent monkey serum.

The challenge doses were sufficient to evoke illness within 12 hr in all animals and to be lethal for 50 to 100% of control animals. Only 60% of surviving controls developed hemagglutinins and 40%, precipitins. After 1 month there was no significant change in the number of controls that were antibody-positive. In animals treated with alpha-globulin from

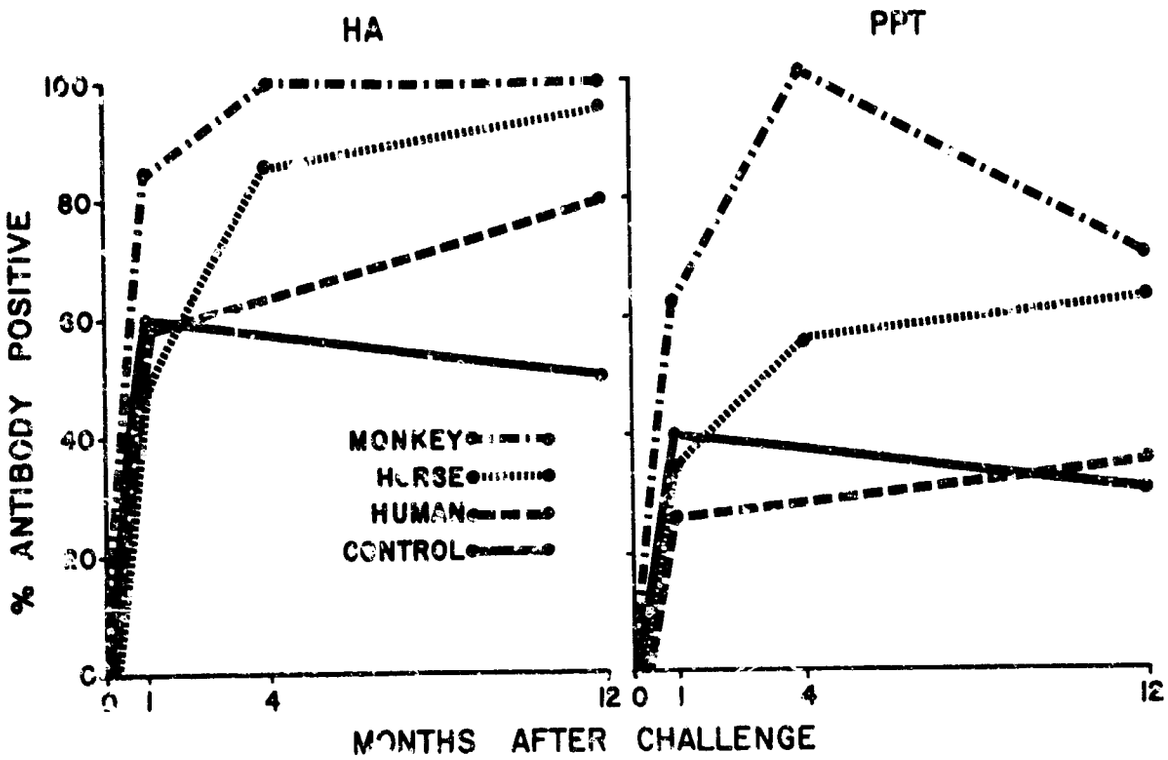

FIGURE 2. EFFECT OF TREATMENT WITH MONKEY, HUMAN OR HORSE ANTITOXIN ON INCIDENCE OF ANTIBODY RESPONSE OF MONKEYS CHALLENGED WITH ENTEROTOXIN.

convalescent human serum at onset of illness, the percent with hemagglutinins was the same as that of the controls at 1 month, but appeared to increase somewhat during the year; the percent with precipitins increased more slowly than the controls. Incidence of responders in monkeys treated with pepsin-digested globulins from hyperimmune horse serum increased significantly throughout the year, and 100% of the animals treated with convalescent monkey serum developed antibody within 3 to 4 months; after this time the number with hemagglutinin response remained the same and the number with precipitin response decreased. All animals that had prechallenge antibody responded within 2 weeks after challenge, regardless of treatment.

Serum therapy apparently delayed development of maximum titer. Essentially all of the control animals that developed antibody had maximum titers within 1 month after challenge. In contrast, only 50% of those that received monkey serum and considerably fewer of those that received sera from other species had maximum titers at this time. Although titer developed more slowly in serum-treated animals, maximum titer was usually higher than that of controls. In general, precipitin titers developed more slowly and decreased more rapidly than hemagglutinin titers.

These data suggested that under proper conditions enterotoxin was highly antigenic but that antibody developed slowly. It is possible that in vivo formation of large aggregates of toxin by immune precipitation and/or protection of the integrity of the toxin molecule in immune aggregates resulted in increased antigenicity. Slow rate of rejection of homologous antitoxin, thereby prolonging the presence of immune aggregates, may have been a factor in the greater effectiveness of monkey antitoxin.

The number of animals responding to enterotoxin and the degree of antibody response were also related to challenge dose and to previous experience of the animals. In Table IV the percent of animals developing a significant hemagglutinin or precipitin response within 2 weeks after challenge with increasing doses of toxin is summarized. Dosage ranged from < 0.1 to 1,000 µg/kg. Animals are grouped on the basis of prechallenge experience and of prechallenge antibody levels. Results for combining activity were essentially the same as those for hemagglutinins.

In general, a challenge dose of 10 µg/kg was required to evoke a significant hemagglutinin response, and 100 µg/kg, a significant precipitin response, in 50% of the nonexperienced, antibody-negative monkeys, whereas 0.1 µg/kg was sufficient for experienced animals. In nonexperienced animals antibody appeared only after overt illness, but in experienced monkeys the response was somewhat better in the absence of illness. Essentially all monkeys challenged with 100 µg/kg or more showed an increase in antibody. At lower challenge doses the antibody response depended on challenge dose experience and amount of prechallenge antibody. Percent reactors increased with dose; at the same dose, with experience; and the higher the prechallenge titer, the greater the dose required to evoke a significant response.

TABLE IV. PERCENT OF MONKEYS WITH SIGNIFICANT ANTIBODY RESPONSE 2 WEEKS AFTER CHALLENGE WITH SEB

PRECHALLENGE		ANTIBODY RESPONSE % OF MONKEYS BY CHALLENGE DOSE (μg/kg)					
Exposures	Antibody	< 0.1	0.1	1.0	10	100	1000
None	No HA	0	15	20	0[a]	(100)[b]	
1 or 2	No HA	0	(100)	80	(50)[a]		
	Low HA	0	60	100	100	100	90
	High HA	0		30	75	100	100
None	No PPT	0	0	0	0	(50)[a]	
1 or 2	No PPT	0	60	100	90		(100)
	Low PPT	0	(50)	75	100	100	100
	High PPT	0		40	100	(100)	(100)

a. Percent responding increased to 80-100% in 4 weeks.
b. ( ) only 2 animals in the group.

SUMMARY

Data on the serology of SEB indicate that hemagglutination and toxin-combining activity were more sensitive than precipitation as indices of antibody, and that there was a relationship between circulating antibody and resistance to overt effects of SEB. Studies on antibody response suggest that SEb may be highly antigenic but that antibody development may be slow. Some factors that enter into the time of appearance and magnitude of response are challenge dose, previous experience or prechallenge antibody, and antitoxin treatment. These results are derived from studies in which monkeys received a single dose or several widely-spaced doses of unaltered toxin. At the present time it would be unwise to predict the effect of multiple, closely spaced exposures to unaltered toxin or to toxoid preparations, or the response of another susceptible species, such as man.

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MECHANISMS OF PYROGENICITY OF STAPHYLOCOCCAL ENTEROTOXIN B

Frank A. Carozza, Jr., Captain, MC*

At the Commission meeting last year evidence was presented[1/] indicating that pyrogenicity for rabbits is an intrinsic property of staphylococcal enterotoxin B (SEB) and not due to contamination of this material with bacterial endotoxin. Moreover, after injection into rabbits SEB liberates an endogenous pyrogen, which presumably mediates the ensuing fever. Preliminary evidence was also presented that after several daily intravenous (IV) SEB injections rabbits become pyrogenicly tolerant and such tolerance was not associated with a demonstrable humoral protective factor. During the past year studies have been initiated to quantitate more closely the enterotoxin pyrogenic tolerant state and define its mechanism of action in the hope that such knowledge would aid in understanding the mechanism of SEB's toxicity for normal animals.

In order to quantitate more closely these observations it was necessary to obtain an SEB dose-response curve. Because of the wide variation of pyrogenic responses to SEB among individual rabbits, however, this could not be done in the usual manner employing small groups of animals. As can be seen at the top of Figure 1 the 2 animals receiving 1.0 µg/kg enterotoxin IV show markedly different responses. The response at the left is minimal while that to the right is exaggerated. Although not as marked, significant variability in response also occurs when animals are challenged with 10.0 µg/kg. Because of this wide variation in individual pyrogenic response a new system was developed so that the dose-response relationship for any group of animals could be predicted without employing large numbers of rabbits.

On day 0 a group of animals was tested with 1.0 µg/kg SEB IV. Animals with uniformly minimal responses were selected; on day 4, or 3 days later, they were retested with 10.0 µg/kg. All animals showing significant increases in response to 10.0 µg were considered acceptable; the mean response to each of the 2 doses is shown in Figure 2. At the left is shown the composite fever curve for each dose and at the right the mean fever index. In this manner a dose-response relationship for these animals could be established. Moreover, and most important, when rechallenged with 10.0 µg/kg on day 7 the mean responses of these animals was unchanged. The significance of this latter finding is that once the dose-response relationship has been established for a selected group of animals, such animals may be employed experimentally and any change in responsiveness to SEB may be accurately quantitated. In addition this observation demonstrates still another difference between SEB and bacterial endotoxin since even as little as a single injection of endotoxin will confer significant pyrogenic tolerance upon the rabbit at these time intervals.

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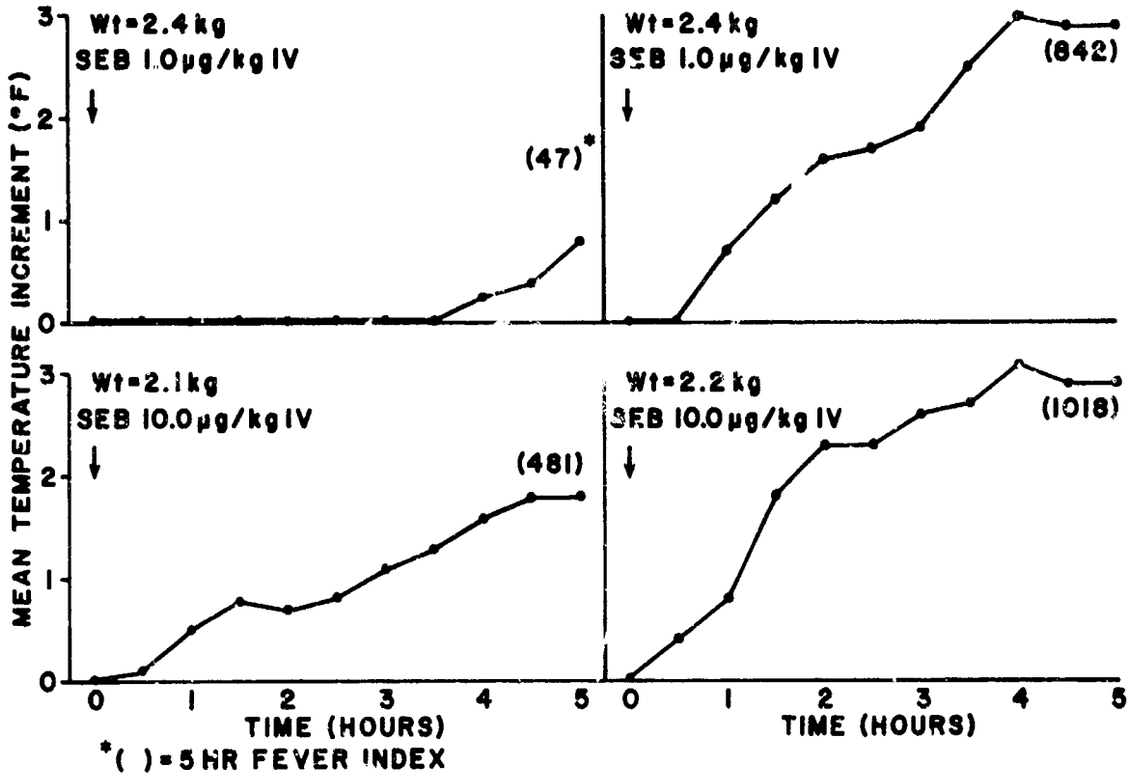

FIGURE 1. VARIATION OF PYROGENIC RESPONSES TO SEB AMONG INDIVIDUAL RABBITS.

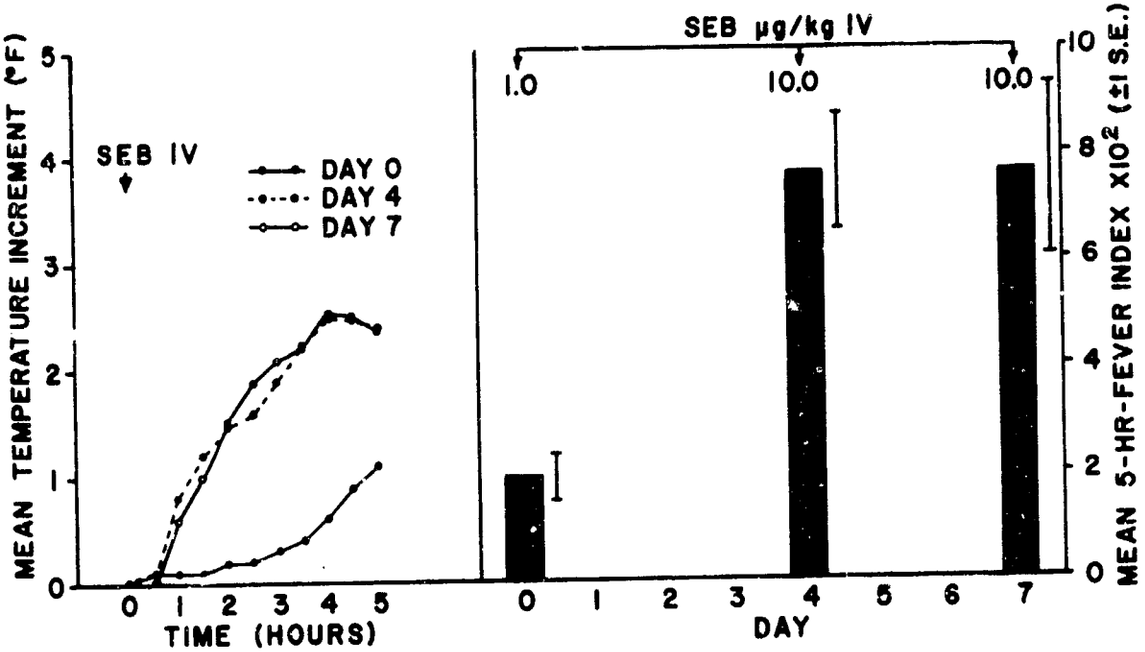

FIGURE 2. PYROGENIC EFFECT OF INTERMITTENT INJECTIONS OF SEB IN 6 RABBITS.

Figure 3 shows the development of a transitory pyrogenic tolerance to SEB in dose-response-selected animals after several single IV injections. On day 0 all animals received 1.0 µg/kg SEB followed by 10.0 µg/kg on the next day; composite fever curves are shown at the left, and mean fever indices to the right. As can be seen, a clear-cut dose-response relationship was readily established in these selected rabbits. Animals then continued to receive single daily IV injections of 10.0 µg/kg for 4 subsequent days. As this time animals reacted less to 10.0 µg/kg SEB than they had initially to 1.0 µg/kg and thus exhibited a high degree of pyrogenic tolerance. Animals were not challenged with SEB on the subsequent 2 days. After this period of rest, however, when rechallenged with 10.0 µg/kg enterotoxin, tolerance partially disappeared. Thus the pyrogenic refractory state or tolerance induced by several injections of enterotoxin is transitory and is partially abolished by 2 days rest.

Figure 4 shows the same procedure repeated in a similar group of animals. A dose-response relationship was established and rabbits rendered pyrogenically tolerant by several daily IV injections of SEB. By day 5 animals were markedly refractory. These animals, however, received no SEB for the subsequent 9 days. Upon rechallenge on the 14th day tolerance had completely disappeared. Thus the pyrogenic tolerance induced by several injections of enterotoxin is completely reversed by 9 days rest. This further demonstrates the difference between SEB and bacterial endotoxin since pyrogenic tolerance to endotoxin is not fully lost in 9 days.

Studies were then initiated to determine the mechanism of the enterotoxin pyrogenic refractory state. Its transient nature suggested that animals might be nonspecifically refractory due to loss of sensitivity of the thermoregulatory centers to pyrogenic stimuli or inability to further mobilize endogenous pyrogen. Thermoregulatory centers appeared to be normal however, since refractory rabbits responded briskly to transferred endogenous pyrogen obtained from the serum of enterotoxin-treated normals. Moreover rabbits made refractory to SEB by several daily injections responded normally to bacterial endotoxin, indicating that the refractory state is not mediated by an inability to mobilize and respond to endogenous pyrogen.

Another possibility was that refractory rabbits might be protected from the pyrogenic effect of enterotoxin by hyperactivity of the reticuloendothelial system (RES). At the left of Figure 5 is shown the dose-response relationship for 10 selected rabbits receiving 1.0 µg/kg followed by 10.0 µg/kg staphylococcal enterotoxin IV. After 5 days rest the same animals were blockaded with Thorotrast(R) and then challenged with 1.0 µg/kg SEB. These RES-blockaded animals now reacted more than initially to 1.0 µg/kg and, indeed the response, was equivalent to that elicited by 10.0 µg/kg. Thus in these animals RES blockade enhanced enterotoxin sensitivity approximately 10-fold. At the right a similar dose-response relationship is shown for a second group of selected rabbits. On the subsequent 4 days each animal received 10.0 µg/kg SEB

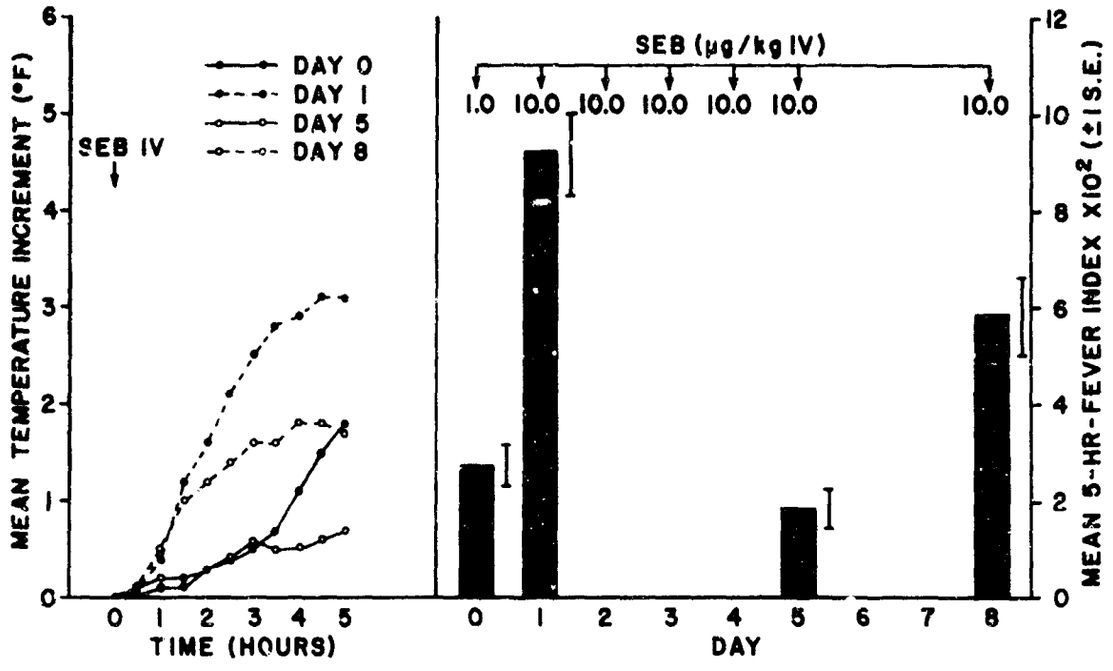

FIGURE 3. DEVELOPMENT OF AN EARLY, TRANSITORY PYROGENIC TOLERANCE TO SEB IN 8 RABBITS.

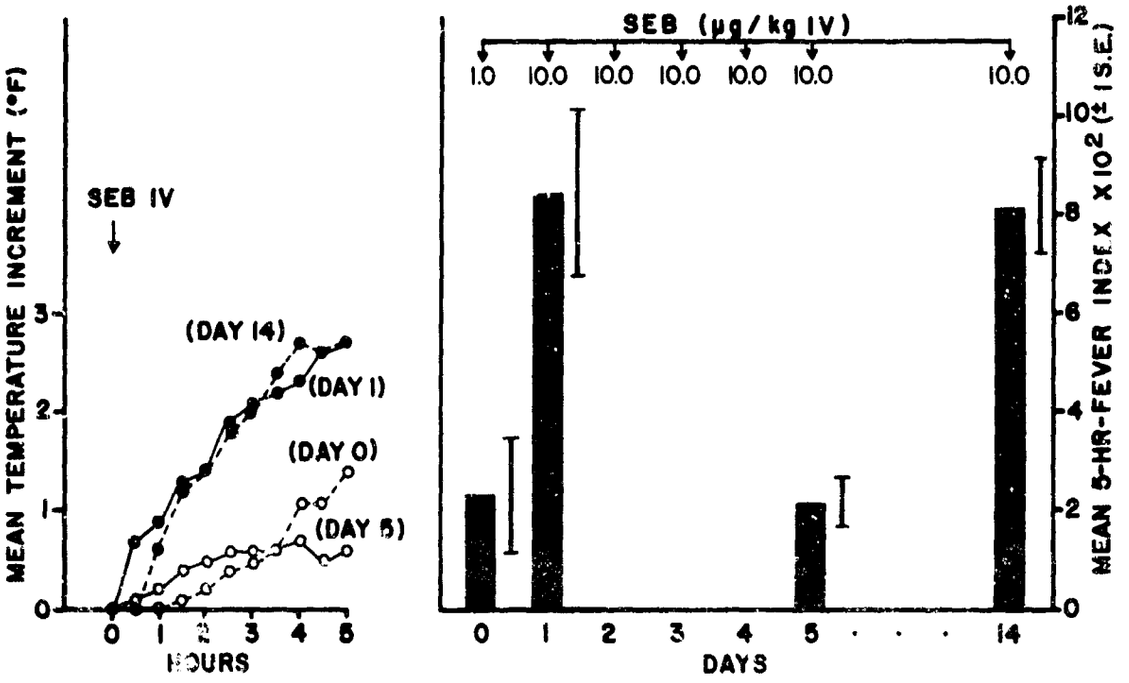

FIGURE 4. COMPLETE REVERSAL OF PYROGENIC TOLERANCE TO SEB 9 DAYS AFTER CESSATION OF INJECTIONS.

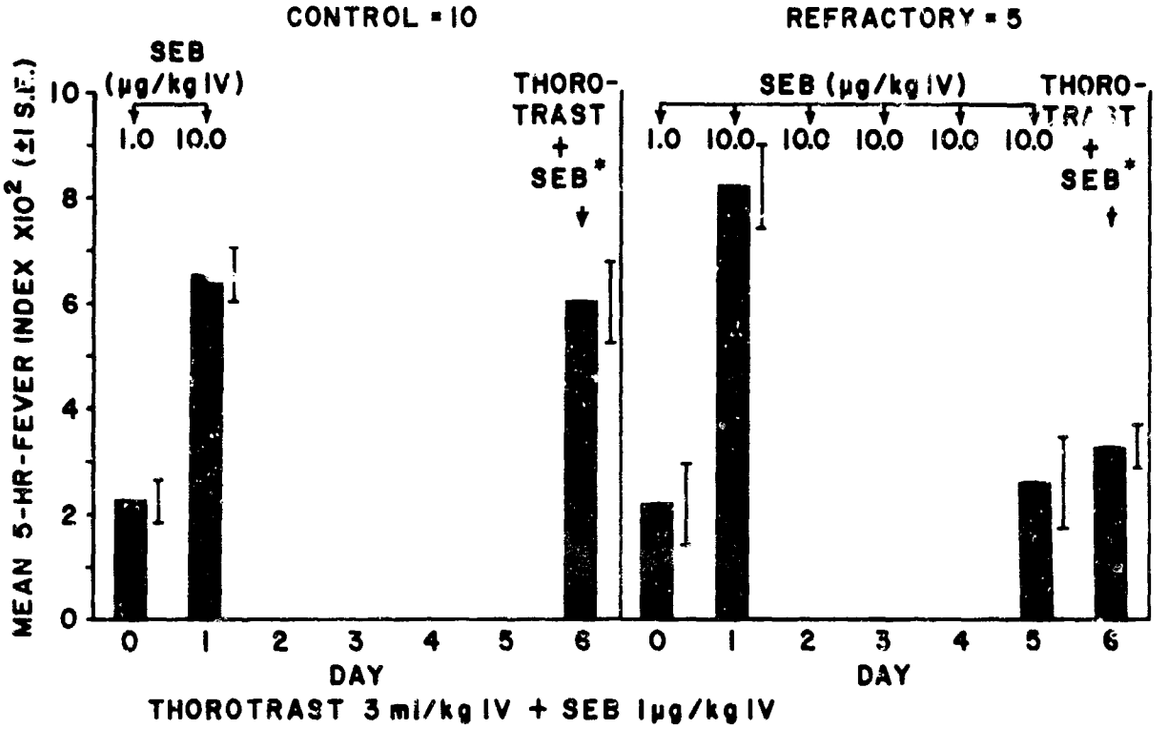

FIGURE 5. EFFECT OF RES BLOCKADE ON SEB PYROGENIC REFRACTORY STATE IN RABBITS.

IV. By day 5 this group exhibited significant tolerance. On day 6 the RES of these tolerant animals were blockaded with Thorotrast(R) and all animals were challenged with 1.0 µg/kg SEB. Again RES blockade enhanced enterotoxin sensitivity 10-fold as this group also reacted to 1.0 µg/kg as they had to 10.0 µg/kg on the previous day. However, when one compares the response of the tolerant, blockaded animals to 1.0 µg/kg with the response of the nontolerant group to the same dose, the bulk of enterotoxin tolerance is retained. Thus, the greater part of the enterotoxin pyrogenic refractory state does not depend upon generalized hyperactivity of the RES.

Another possible mediator of pyrogenic tolerance to SEB is a circulating humoral factor or antibody. No antibody, however, could be detected in control or refractory state sera employing the agar diffusion and tanned sheep cell hemagglutination tests. Nevertheless the possibility of a circulating protective factor not detected by the serologic tests employed remained. An attempt was made therefore to demonstrate such a factor by means of passive transfer of the refractory state. Figure 6 shows that this could not be accomplished. Eleven rabbits were challenged IV with 10.0 µg/kg SEB. Their composite pyrogenic reaction is shown at the left. These animals were given subsequent single daily enterotoxin injections and by day 4 exhibited marked tolerance. On the following day animals were exsanguinated and plasma separated and frozen. Within the next week a dose-response relationship for 1.0 and 10.0 µg/kg doses was established for 8 normal rabbits. Three days later each animal was given 15 ml/kg tolerant donor plasma IV and 18 hr later or on day 7 challenged with 10.0 µg/kg SEB. As can be seen at the far right of the figure the mean fever index after pretreatment with tolerant donor plasma was not significantly different from the control response to the same dose. Thus by the methods employed the enterotoxin tolerant state could not be passively transferred. This once again demonstrates a difference from the endotoxin system in which pyrogenic tolerance can be readily transferred by this method.

Because the enterotoxin pyrogenic tolerant state is transitory, appears specific for SEB and does not appear to be mediated by a protective humoral factor or hyperactivity of the RES, it was felt that pyrogenic tolerance might represent a specific desensitization to enterotoxin similar to that which has been described by Greisman et al[2] for bacterial endotoxin. Thus studies were initiated to determine: (1) if rabbits would develop a local reaction to intradermal (ID) enterotoxin and (2) if such reactivity would diminish as animals became pyrogenically tolerant after several daily injections.

The rabbits' abdomens were shaved and inoculated in each of 2 areas with 100 and 10 µg SEB in 0.1 ml normal saline. Although erythematous lesions appeared in 24 hr, a maximal difference between the 2 doses was only apparent at 48 hr. Figure 7 shows the 48-hr lesions in a normal animal. At the left is a large erythematous, nonindurated response to

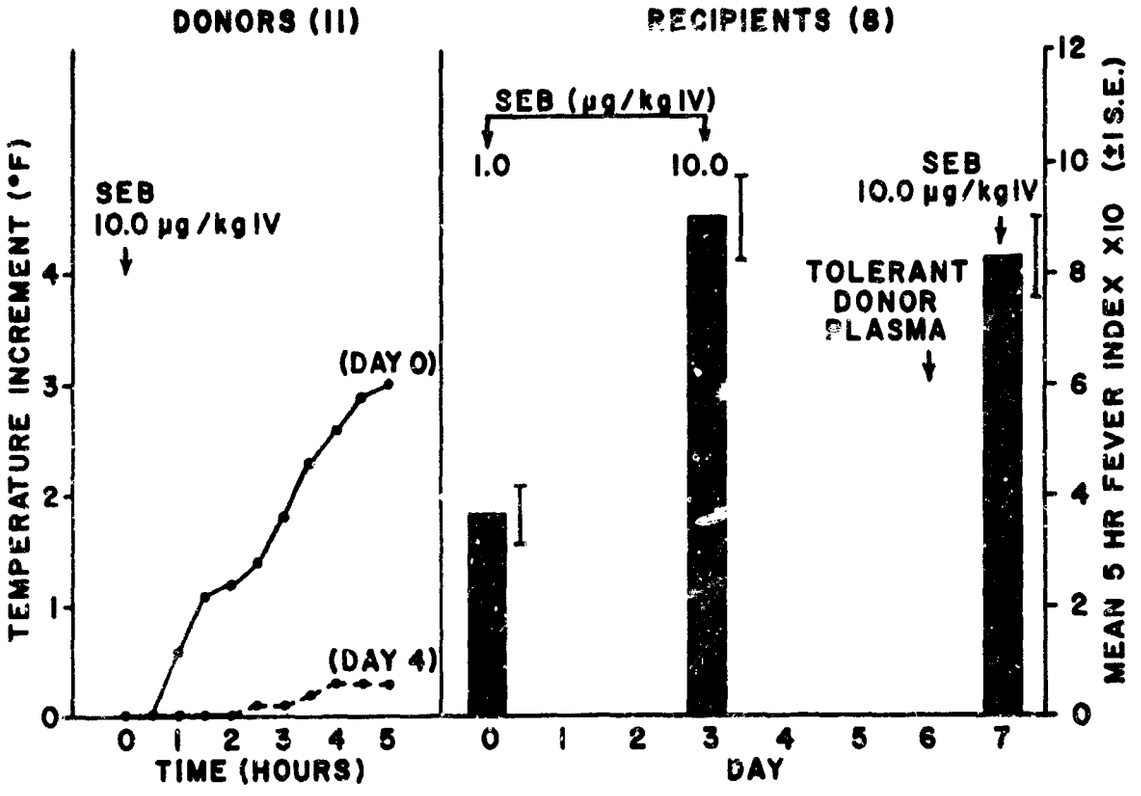

FIGURE 6. PYROGENIC EFFECT OF SEB IN RABBITS PRETREATED WITH PLASMA FROM TOLERANT DONORS.

100 µg SEB. On closer examination a few petechiae can be seen scattered through the affected area. On the right is a similar but smaller response to 10 µg.

Figure 8 shows that the microscopic lesion consisted of an infiltrate of eosinophiles, lymphocytes, plasma cells and mononuclear cells with scattered polymorphonuclear leukocytes. There was also much nuclear debris and edema in the dermis and subcutaneous tissue. Although gross induration was minimal to absent in these lesions the microscopic findings were compatible with a hypersensitivity reaction of the delayed type.

An attempt was then made to desensitize rabbits to the dermal enterotoxin reaction by several IV injections. Eight normal animals received 100 and 10 µg SEB in each of 2 abdominal sites. The area of each reaction was calculated and the mean reaction to each dose is shown at the left of Figure 9. There was a significant difference in the response obtained for each dose. Each of second group of animals was pretreated with 10 µg/kg SEB IV daily for 5 days and tested ID with 100 and 10 µg enterotoxin. As can be seen at the right of Figure 9 a significant diminution in the size of the dermal response occurred. Indeed animals pretreated with IV SEB reacted to 100 µg ID enterotoxin as normals receiving only 10 µg. There was only a slight decrease in reaction to 10 µg. Thus rabbits can be made partially refractory to the enterotoxin skin reaction by several daily IV injections, and such refractoriness occurs at a time when the enterotoxin pyrogenic refractory state is maximal.

SUMMARY

Rabbits became pyrogenically refractory to staphylococcal enterotoxin B (SEB) after 4 single daily IV injections. This state was transitory and disappeared completely within 9 days if animals are not repeatedly rechallenged. The state appears specific for SEB since refractory animals react normally to endotoxin and endogenous pyrogen.

The refractory state is not mediated by a demonstrable circulating humoral protective factor nor generalized hyperactivity of the RES. The occurrence of the state can be correlated with a significant diminution in skin sensitivity to enterotoxin.

These data are compatible with the thesis that the fever produced in rabbits by SEB is mediated by a hypersensitivity reaction possibley of the delayed type.

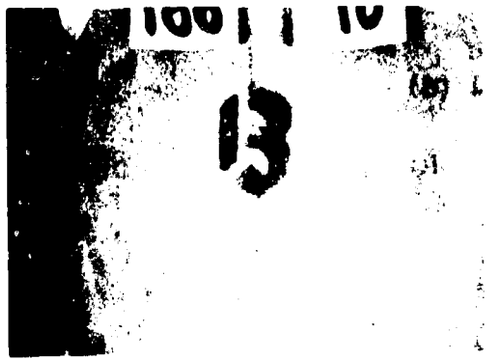

FIGURE 7. 48-HR REACTION OF A RABBIT. INTRADERMAL SEB (100 µg on left, 10 µg on right)

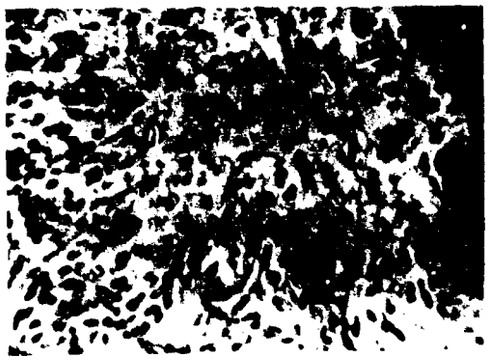

FIGURE 8. SECTION OF SKIN FROM 100 µg REACTION ABOVE H&E 900 X

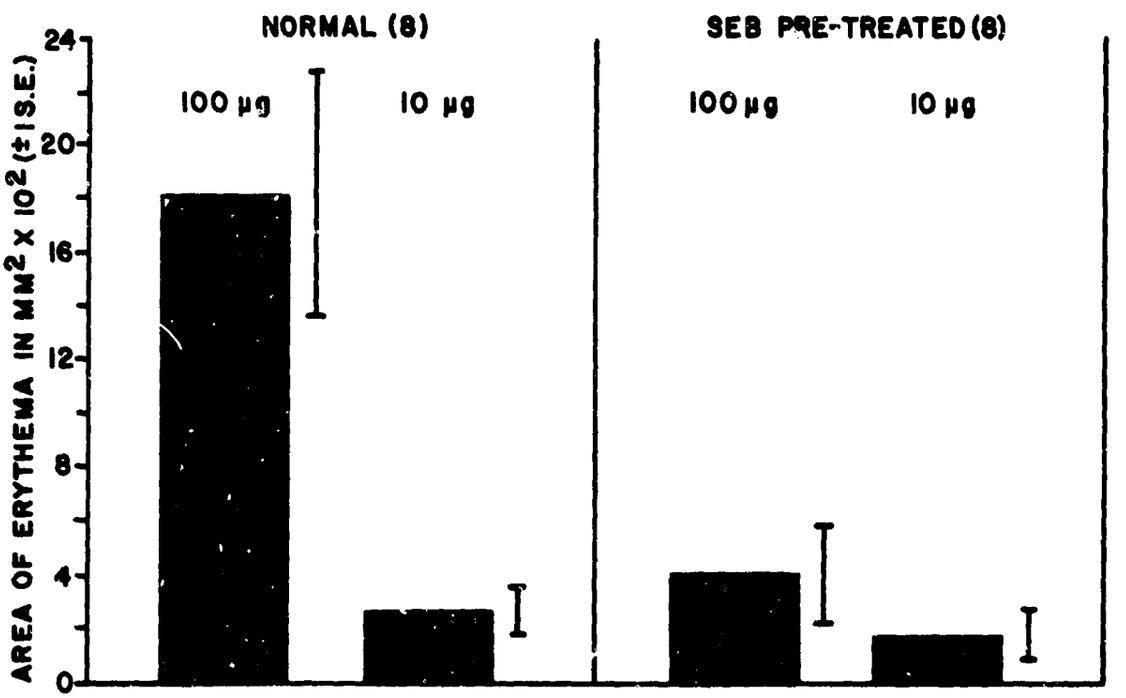

FIGURE 9. 48-HR REACTION TO INTRADERMAL SEB IN NORMAL RABBITS & THOSE PRETREATED WITH IV SEB.

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RECENT STUDIES ON ANTHRAX TOXIN

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and Anne Buzzell, Ph.D.*

Work on anthrax toxin in other laboratories has been concerned largely with studies on its three recognized components, separately isolated and purified. Each of these fractions is nontoxic when injected alone, but active in appropriate recombinations. One component, which appears to be identical with the immunizing, protective antigen (PA) is essential for demonstration of activity in all recombinations. The component which in combination with PA produced edematous lesions in the skin of guinea pigs or rabbits after intradermal inoculation, is called edema factor (EF). The other, which when combined with PA causes death in certain species after intravenous (IV) challenge, is called lethal factor (LF).

For several reasons, it seemed desirable to attempt to concentrate culture filtrates and purify that portion responsible for toxic activity without prior fractionation into its separate components. We have been working toward this goal over the last several years. In a previous report[1] we described a simple ultrafiltration method for concentration of whole crude toxin. Small diameter dialysis tubing is used as the filtering membrane, in a continous flow system under negative pressure. All detectable biological and antigenic activity remains in the dialysis sac after processing. This ultrafiltrate residue, harvested by "back-washing" with Tris buffer, has been designated UFR. In 1964 we reported[2] the results of initial studies, designed to characterize the biological activity, storage stability and immunogenicity of this material. At last year's meeting, one of us (A.B.) presented work[3] on some of the biophysical characteristics of UFR.

Initial studies on purification and biochemical characterization of the concentrated toxin had to be interrupted at an early stage. This work has been resumed only recently, but it now appears that it will be entirely phased out of our research program within the next few months. For this reason I should like not only to describe current studies on the toxin but also to review briefly other work that seems pertinent to our present interpretation of available data on the nature of this material.

Results of studies on UFR employing electrophoresis on paper, cellulose and gel discs all indicate greatest resolution under conditions best for study of peptides and small proteins. On disc electrophoresis, at least 16 proteins staining bands were observed in freshly prepared or freshly thawed frozen preparations. Upon heating at 35 C for 30 min to 1 hr the number of bands increased initially, but there was no immediate decrease of rat lethal activity. After 4-6 hr at this temperature, the number of protein staining bands decreased and the amount of ninhydrin staining

* U. S. Army Medical Unit.

material increased. Biological activity rapidly decreased upon heating for > 6 hr and was entirely lost after 24 hr. Similar changes in disc pattern and biological activity occurred upon storage at 4 C, but much more slowly.

Immunodiffusion of longitudinal sections of the acrylamide discs against antiserum prepared by hyperimmunization of a burro, with spore vaccine, showed that many of the 16 bands seen in the stained disc shared common antigenic determinants. At least 3 components, of which one was protective antigen, formed continuous lines of immune precipitate down the length of the disc. The presence of 3 or 4 antigenic components identifying throughout the disc pattern was confirmed by reacting transverse sections of the column against burro antiserum in identity plates. These findings suggested that at least 3 components in UFR were in various states of polymerization, were complexing with other substances, or were extensively fragmented.

The UFR was examined by the standard agar gel immunoelectrophoresis[4/] method; test samples were reacted against the standard burro antiserum and also against antisera prepared in rabbits by immunization with repeated doses of UFR, combined with Freund's complete adjuvant. Only 5 or 6 antigens, of which 2 were major, were demonstrated in studies with the burro antiserum. With the rabbit antiserum about 12 lines of equal intensity developed. All antigens detected by burro antiserum were also demonstrated with rabbit, but 5 or 6 other antigens in UFR, reacting with rabbit antisera, were not detected by the serum of the burro immunized with the spore vaccine. A comparison with partially purified protective antigen showed that the PA fraction of UFR migrated in agar gel in a position comparable to serum β_2 globulins and that other components migrated in positions anodic to PA.

In preliminary experiments with ion exchange columns, it was found that the UFR proteins were bound in varying degrees to DEAE-cellulose in the hydroxyl, chloride, bicarbonate, or acetate forms but not in the phosphate form. The proteins were eluted by increasing concentrations of phosphate, acetate, or bicarbonate buffers in a neutral pH range. The concentration of buffer at which each toxin component was eluted was the same for all buffers used.

A column equilibrated and eluted with ammonium acetate buffers was chosen as the reference preparative column because of better yields, higher activity of products and the convenience of use of a volatile buffer.

In Figure 1, a chromatogram of UFR on such a column is shown. The components eluted in the peaks were identified by reactions with antibody and biological activity on recombination. EF was located in peaks I and III; some PA also appeared in III but was found mainly in peak IV, LF was identified in peaks VI and VII. The proteins in peaks II, V, and VIII

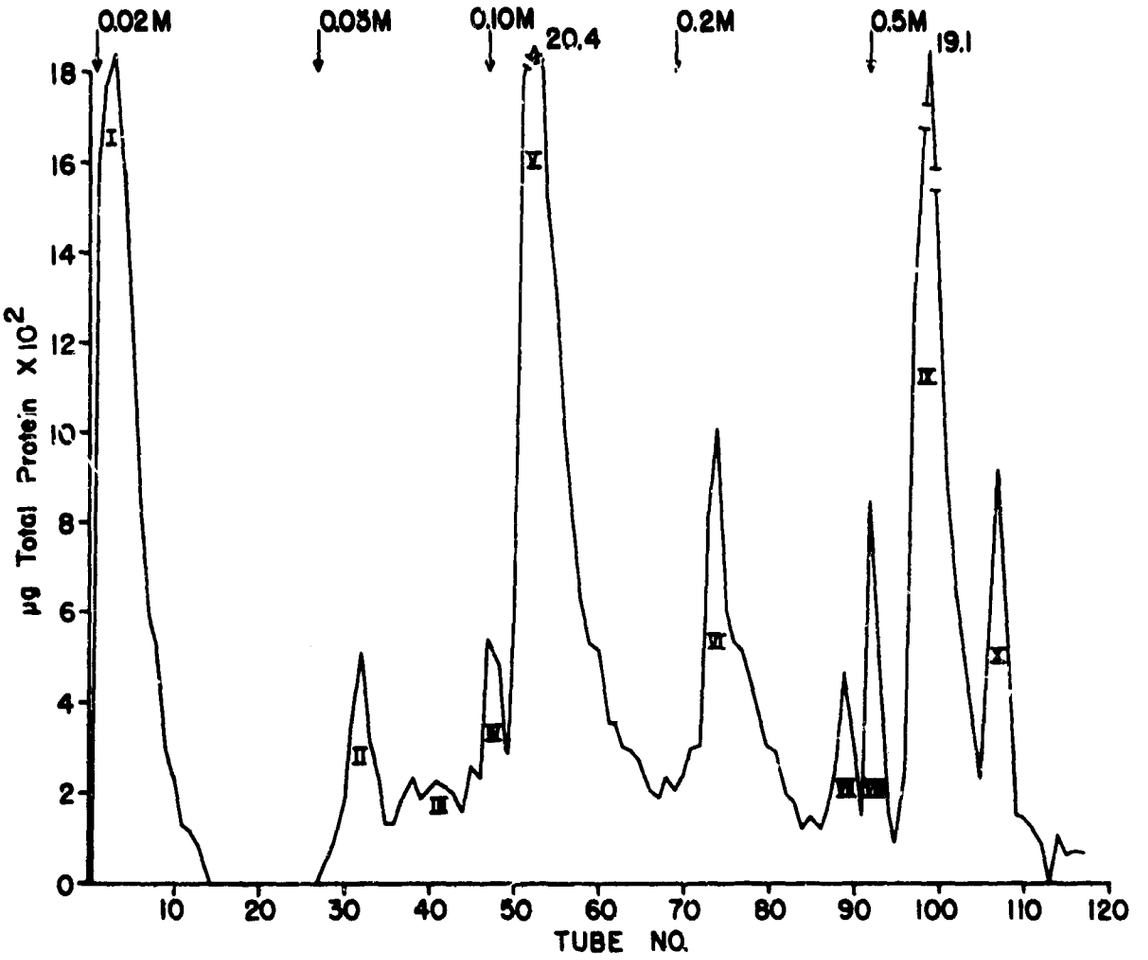

FIGURE 1. CHROMATOGRAPHY OF 2L CRUDE CULTURE FILTRATE ON DEAE-CELLULOSE ELUTED WITH AMMONIUM ACETATE BUFFER GRADIENT (pH7.0).

have not been identified. Protein recoveries, as measured by the Lowry method, averaged 82%, and recoveries of biological activity were approximately 80% as measured by the rat lethal assay.

In other chromatograms some PA is bound to LF. The complex was easily dissociated by dilution, however, and the 2 components were resolved by rechromatographing on the same type column. This procedure yielded a fraction with LF activity, which gave a single band on gel diffusion against spore or UFR antiserum. It showed a single major band on disc electrophoresis but had a minor, nonantigenic chromagen-type contaminant. Protective antigen from peak IV was further treated by rechromatography with an ECTEOLA-acetate system. PA was not bound on ECTEOLA and was eluted free of other detectable components. This fraction showed a split band on disc electrophoresis and a single band when reacted against burro or rabbit antiserum.

These preparations may be contaminated by other, as yet undetected, substances. Crude or purified PA preparations as well as UFR degrade in a temperature dependent manner. We have found in both PPA and UFR, proteinase activity which has been quantitated by the Azocoll assay of Oakley et al.[57] The relationship of this proteinase to that detected by other workers using different substrates is unknown. The column preparations may contain some enzyme contamination.

For comparative studies, the same chromatographic procedures have been applied to crude culture filtrates. The crude material, diluted 1:9 with distilled H_2O, was applied to the DEAE column and the column was eluted with the same program. Results are shown in Figure 2. The position of major peaks, and the components identified in them, were almost identical with those of a UFR chromatogram with the following exceptions: In no case did LF have detectable PA associated with it; both PA and EF were detected only in a single peak. The total yield of protein per liter of crude filtrate was twice that of UFR and 80% of the starting biological activity as calculated from the rat lethal assay was recovered.

Although complete separation of EF from other components has not been achieved by the present methods, preliminary work with other chromatography programs indicates that it is possible to do so. It definitely appears that the 3 components of toxin are quite readily dissociated from each other by dilution or the very gentle treatment involved in chromatography. Even on preparative columns no single peak contained all components necessary for toxic activity. It seems doubtful that a single, even loosely bound, toxic entity exists as such in either the UFR or crude culture filtrates.

After the biologically active components are eluted from the preparative column, a chromogenic fraction of brown coloration remains. It can be eluted in a relatively pure state by 0.1 N NaOH. The chromophore group of this substance is bound to a protein with an isoelectric point at pH 3.8, is easily hydrolyzed from the protein by mild acid conditions, is significantly soluble in ether, and has a strong ultraviolet (UV) absorbing peak

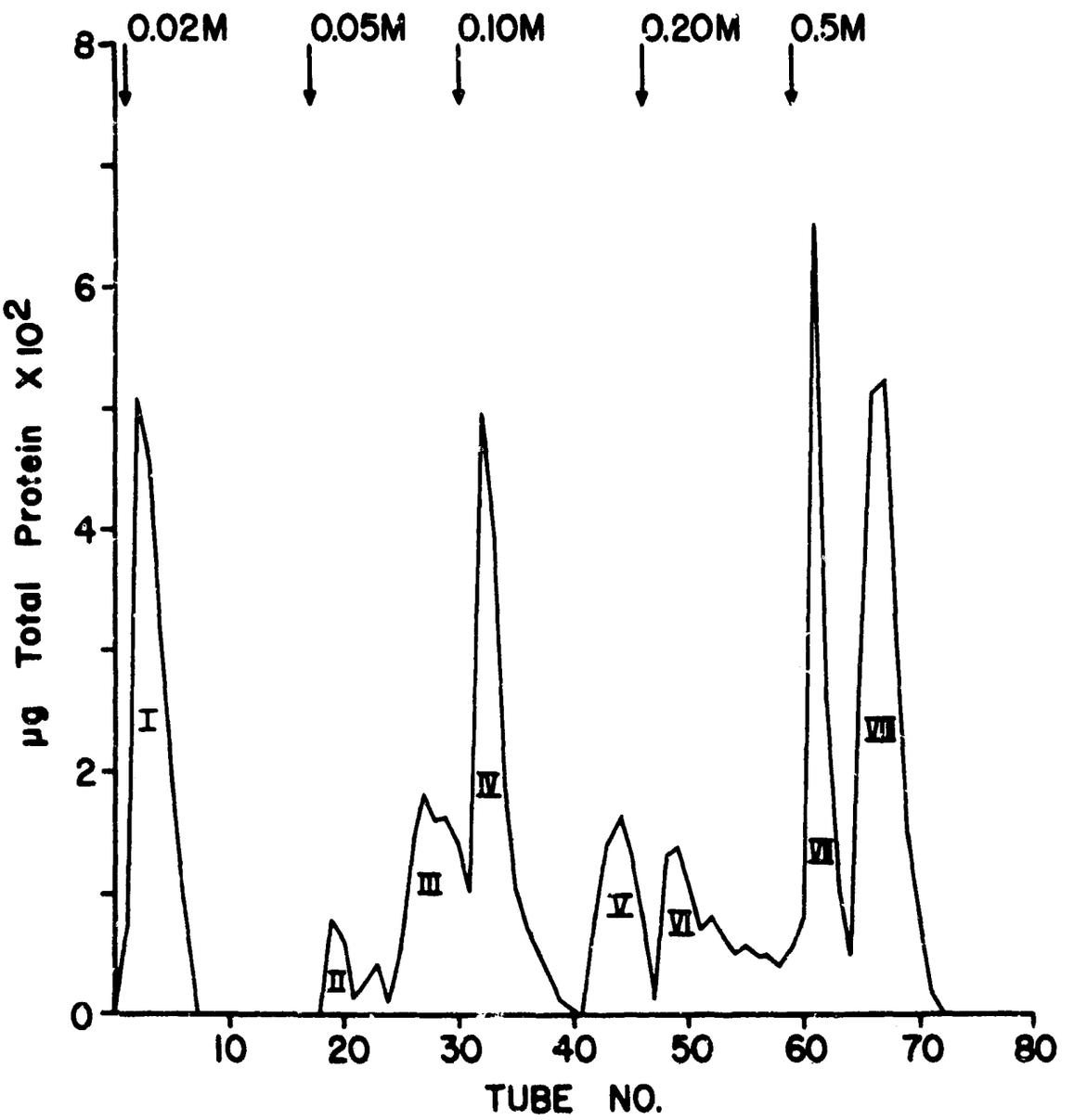

FIGURE 2. CHROMATOGRAPHY OF 1 ml UFR (719 X) ON DEAE-CELLULOSE ELUTED WITH AMMONIUM ACETATE BUFFER GRADIENT (pH 7.0).

at 260 mμ. The substance is nonantigenic; its spectrum is unaffected by ferric ions; and the pigmentation is increased by reduction with cysteine. This substance is of interest because, although it varies from batch to batch, it can represent as much as 40% of the total protein recovered; it is a ubiquitous contaminant in earlier protein fractions; its high UV absorption at 260 mμ may be responsible for the peak at this wave length of whole UFR; and it may be related to the material absorbing at 260 mμ in EF preparations described by the British workers.[6] It appears to be different from the red-pink pigment described by other investigators.

Other acid-hydrolyzable, ether-soluble compounds, as yet unidentified, are present in UFR and in isolated fractions. One compound whose spectrum shifts on addition of Ca ion has been observed. Another compound in peak II of a crude culture filtrate chromatogram is inactive when protein bound, but reduces strongly when hydrolyzed. The significance of these materials is as yet unknown.

One of the considerations stimulating attempts to concentrate active material in crude toxic filtrates without prior separation into recognized components, was the question of whether or not toxic activity could be isolated as a single entity. It seemed possible that if the organism produced toxin as a single molecule composed of several components which were weakly bound, the filtration through sintered glass might contribute to its fragmentation. The work reported here appears to support the view that "toxin" does not exist as a distinct chemical entity *per se*. Furthermore, whether or not this material should be called "a toxin" or even "toxins," in the sense of the word as generally used, is open to debate. LCDR Gaspar[7] in our laboratory first demonstrated that injections of PA followed by LF within 1 hr still caused death in the rat. When LF was injected first, followed by PA, the time of the 2nd injection could be extended for $\geq$ 4 hr. Molnar and Altenbern[8] who confirmed and extended these studies, also demonstrated that the lethal effect of fully toxic mixture of PA and LF could be prevented by prior injection of PA but not of LF. The results of a single experiment in our laboratory using PA tagged *in vitro* with I^{131} indicates that this material after IV injection is rapidly cleared from the blood stream of mice. The work of Molnar and Altenbern,[8] indicates that this is also true in the rat, and that LF can be detected in the circulation for a much longer period of time. These studies suggest that the components necessary for demonstration of toxicity may be acting in sequence (at least in the rat) rather than in true combination.

Our work indicates considerable heterogeneity of components in the UFR. It seems likely that there is significant protein-protein interaction of materials in the preparations concentrated by the method used. There is evidence to suggest that active components of UFR polymerize in what now appears to be a quite regular fashion. The work of one of us (A.B.) is consistent with the hypothesis that the protective antigen component of UFR forms relatively stable, even numbered aggregates by a process of

successive dimerization. Lethal factor seems to be of dimensions closely similar to PA, although chromatographic studies indicate that it is chemically distinct. Single molecules of LF appear to attach to dimers and tetramers of PA but not to single particles. However, as noted earlier, results of work with chromatography suggest that the attachment to LF and PA is readily reversed and probably plays little part per se in the mechanism of toxic action.

Some preliminary experiments indicate that treatment with UFR with 2 M urea, results in complete destruction of lethal activity for rats and most of the antigenic activity of the sample. Disc electrophoresis of urea-treated UFR showed an entirely new pattern of bands; chromatography resulted in a new elution pattern. These results suggest that very weak (or few) hydrogen bonds may be involved in the integrity of one or more components or in the structure of polymers.

UFR is a highly complex and dynamic mixture. In all honesty we must admit that our work has provided more questions than answers. About the only thing we can say with complete certainty is that this is an extremely interesting and challenging material with which to work, albeit at times a chemist's and biologist's nightmare.

SUMMARY

Literature on the nature of the three components of anthrax toxin and conditions affecting toxigenesis was reviewed. Recent studies on the biochemical and biophysical properties of toxic filtrates concentrated by an ultrafiltration method were presented. Available evidence suggest that: (1) "toxin" is not a single chemical entity per se but that its three recognized components exist separately and in the uncombined state in culture filtrates; (2) toxic filtrates are highly complex and dynamic mixtures in which considerable protein-protein interaction, proteinase activity and polymerization of antigenic components occurs; (3) previously unrecognized oxidation-reduction systems and materials of high chelating activity are present in the preparations studied. The biological significance of these components is not known.

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INFLUENCE OF PNEUMOCOCCAL INFECTION ON A HOST ENZYME SYSTEM

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For over 60 years it has been known that certain enzymes in microorganisms are formed only in the presence of their specific substrates. Knox et al[1] reported that Karstrom named this phenomenon enzymatic adaptation; it has been the subject of a great deal of experimentation, speculation and a recent Nobel prize. With the advent of more refined methods of tissue enzyme analysis has come the knowledge that numerous animal tissue enzymes are also subject to adaptive responses similar to those observed in microorganisms. Alterations of enzymes or other specific proteins within tissues have been observed as a manifestation of a number of host adaptive responses. These alterations have been correlated with changes in diet, aging,[2] and even time of day.[3] Likewise various disease processes have also been studied and shown to induce metabolic adaptations. However host enzymatic changes secondary to the infectious process have not been extensively studied. It therefore seemed appropriate to investigate changes in tissue enzymes in infected animals to learn more regarding early biochemical or metabolic alterations within the host during the infectious process.

The hepatic enzyme, tryptophan pyrrolase, was selected by us for specific detailed study for a number of reasons: (1) changes in the activity of this enzyme have been observed by several groups after induced infection or endotoxemia in laboratory animals, (2) the enzyme has an extremely rapid turnover rate, and (3) it appears to be the rate-limiting requirement for a vital metabolic pathway, (4) its activity is partially related to adrenal hormone concentrations.

The half-life of this enzyme has been estimated to be less than 2 hr, as opposed to average total hepatic protein synthesis which varies from 2 to 3 days. The enzyme is rate-limiting for the conversion of tryptophan to formylkynurenine which leads in turn to the eventual biosynthesis of nicotinamide.

Knox and Auerbach[3] in 1954 showed that adrenal steroids were capable of inducing marked increases in hepatic tryptophan pyrrolase activity in rats within 2 to 3 hr. They also showed that the adrenalectomized state was associated with reduced enzyme activity. In other studies, tryptophan, the substrate, was shown to increase enzyme activity within 2 hr of administration. However, corticoid and the substrate induced changes in enzyme activity appeared to result from different mechanisms. Schimke and co-workers[4] reported that adrenal steroids increased tryptophan pyrrolase activity by increasing the rate of m-RNA synthesis. In contrast, the increased enzyme activity attributed to tryptophan administration resulted not from new enzyme synthesis but rather from a stabilization of previously formed enzyme;

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this stabilization resulted in a reduction in the degradation rate. Various cofactors and metal ions such as hematin, Cu and Mn have been shown to increase tryptophan pyrrolase activity. In addition to the protein synthetic inhibitors such as actinomycin and puromycin that are associated with reduced tryptophan pyrrolase activity, Rivlin and Knox[5] observed that bovine growth hormone given to mice markedly reduced enzyme activity within several hours of its administration. Therefore adrenal steroids and growth hormone have antagonistic actions relative to tryptophan pyrrolase activity.

Infection-produced alterations in tryptophan pyrrolase activity have been observed by several groups. Berry and Smythe[6,7] have reported the influence of bacterial endotoxin on mouse tryptophan pyrrolase activity. Early stimulation followed by a variable period of inhibition of enzyme synthesis was observed. Pretreatment of mice with cortisone seemed to counteract the inhibitory influence of endotoxin on the enzyme. These data prompted Berry to speculate that the glucocorticoids were beneficial in endotoxemia because of their ability to increase tryptophan pyrrolase activity and thereby maintain a very vital biosynthetic pathway.

Kong and Smith[8] reported an inhibition of tryptophan pyrrolase activity within 1 hr of massive infection with Staphylococcus aureus. However, experiments testing the influence of adrenal steroids were not done by these workers.

In a previous presentation to this group Lust[9] reported that generalized infection with type IA-5 pneumococcus in mice was associated with early increases in hepatic protein synthesis as evidenced by increased incorporation of C^{14} leucine into microsomal protein. It therefore seemed desirable to study the influence of generalized pneumococcal infection on tryptophan pyrrolase activity in an attempt to determine if this particular single hepatic protein with known rapid kinetic changes, might serve as an appropriate model system to study mechanisms of altered protein synthesis during acute infection.

Male CD-1 mice weighing 12-16 gm were used in all experiments. Infection with a virulent type IA-5 pneumococcus was accomplished subcutaneously and two different dose levels were used. Control animals received an equivalent volume of culture media and were handled in a similar manner to infected mice. A small inoculum of pneumococcus containing 50-100 organisms given subcutaneously in the back was generally lethal within 30-40 hr. Clinical illness characterized by ruffled fur, anorexia, and reduced physical activity was apparent within 16-24 hr. A large inoculum of 5,000,000 to 10,000,000 organisms administered subcutaneously was also used in selected experiments. With the large inoculum, clinical illness was apparent within 8-12 hr and death occurred within 16-26 hr. A transient bacteremia was present within 30 min in both infections. A septicemic pattern became prominent shortly prior to the onset of clinical symptoms.

Plasma corticosterone determinations using a fluorometric technique were obtained in both varieties of infection. Tryptophan pyrrolase activity of liver homogenates was determined by the spectrophotometric method of Knox and Auerbach.[3] All data to be presented represents 6 or more pools of 3 mice at each time period with the mean ± 1 SE depicted.

In Figure 1 are shown serial plasma corticosterone determinations following pneumococcus inoculations. A biphasic response is suggested after infection with both the large and small inocula. Note however that steroid levels in the more fulminate infection become elevated earlier and reach higher levels than that observed with the small inoculum.

The effect of the small inoculum of pneumococci on tryptophan pyrrolase activity determined serially over the initial 21 hr of infection is shown in Figure 2. A circadian rhythm in the activity of this enzyme was recognized for the first time in our preliminary studies. This made it imperative to match each point of data in infected animals with an equal number of control observations. It may be seen that within 2 hr of inoculation a significant increase above the control is apparent. Enzyme activity remains elevated throughout the initial period of infection but then returns to normal within 15 hr. Figure 3 shows the effect of a large inoculum of pneumococci on enzyme activity. Except for a period of reduced activity apparent at 6 hr post-inoculation, values were generally indistinguishable from controls. The initial increase in enzyme activity seen with the small inoculum is not present in this more serious infection.

In an attempt to learn more regrading the mechanism by which a small inoculum of pneumococci could produce increased enzyme activity within 2 hr, a series of related experiments were performed. Various adrenocortical relationships were investigated during these studies. Shown in Figure 4 are 3 pairs of experiments. To the left is a comparison of the enzyme activity in intact normal controls and in intact mice infected 2 hr earlier. The infection-produced increase in enzyme activity may again be seen. In the middle is shown a similar comparison except that mice had been adrenalectomized 5 days earlier. The control value is significantly reduced in agreement with observations of other workers. However, an infection-related increase in enzyme activity failed to occur. On the right is another series of experiments in adrenalectomized mice which received replacement therapy with cortisol, designated Compound F, in divided doses which totaled 0.01 µg daily. Although physiologic replacement increased control activity above normal, there was no additional increase as a result of infection. One must therefore conclude that the presence of intact adrenocortical function is a requirement for the observed increase in tryptophan pyrrolase activity which occurs within 2 hr of infection. Furthermore the steroid influence must be more than permissive, since infection of adrenalectomized mice on an adequate replacement schedule of cortisol was not associated with any additional change in enzyme activity.

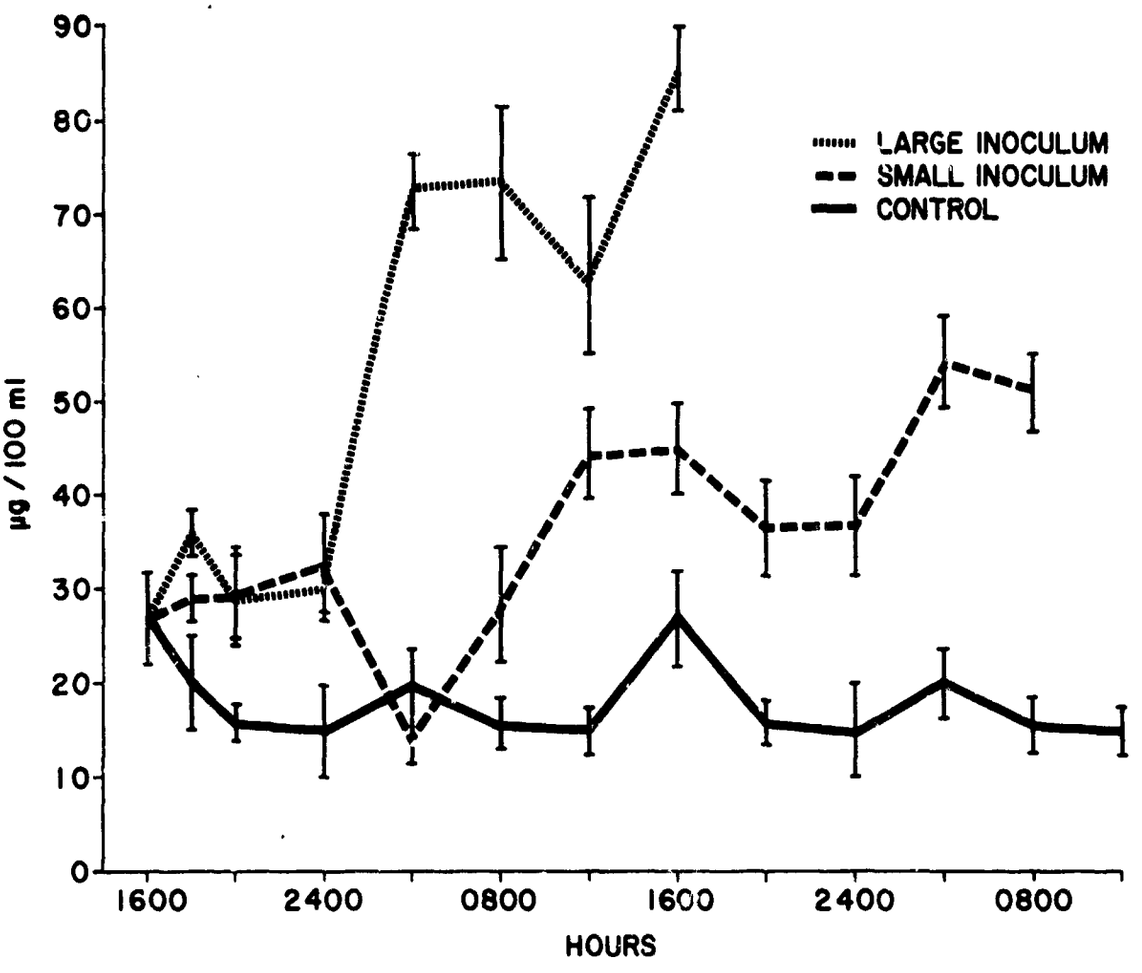

FIGURE 1. PLASMA CORTICOSTERONE IN PNEUMOCOCCAL INFECTION IN MICE.

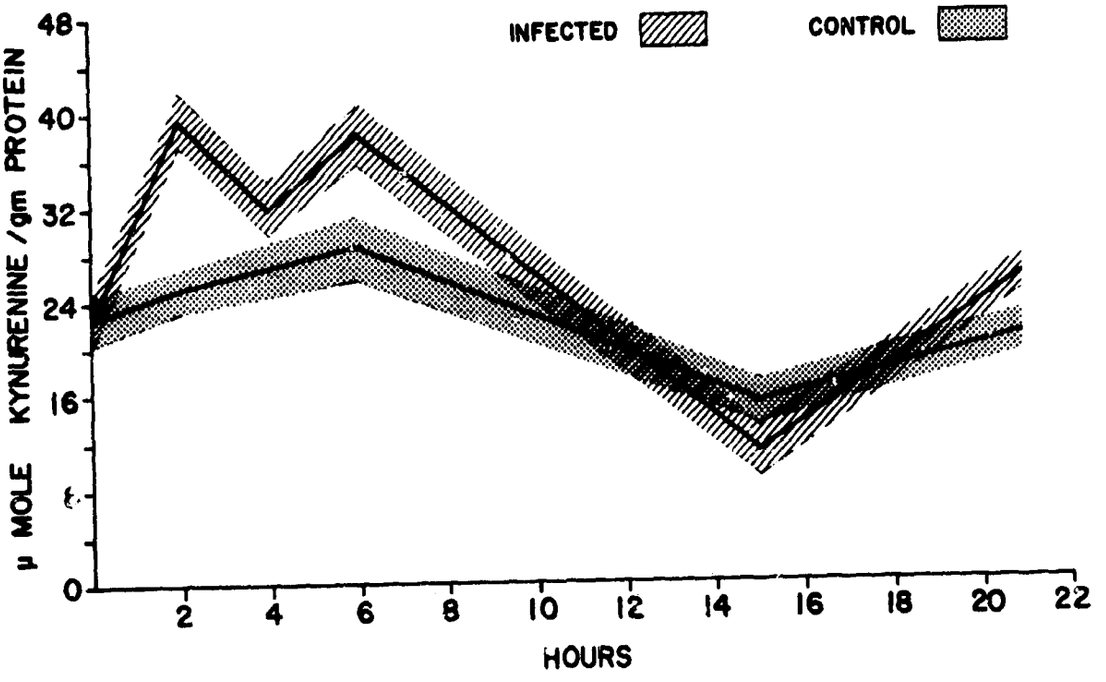

FIGURE 2. TRYPTOPHAN PYRROLASE ACTIVITY IN PNEUMOCOCCAL INFECTION (SMALL INOCULUM).

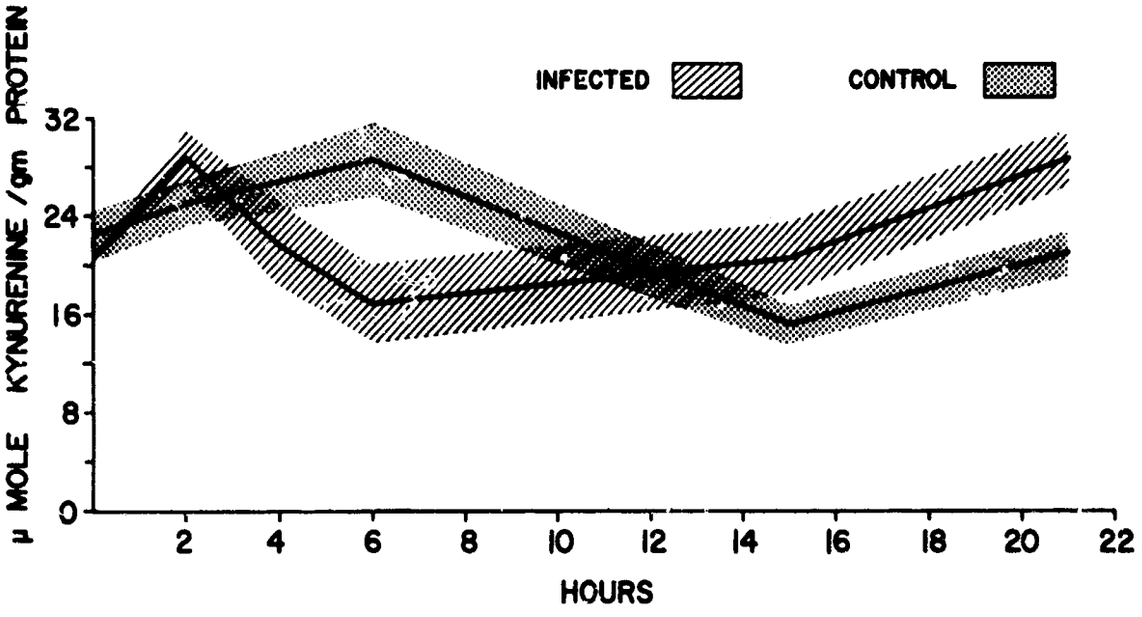

FIGURE 3. TRYPTOPHAN PYRROLASE ACTIVITY IN PNEUMOCOCCAL INFECTION (LARGE INOCULUM).

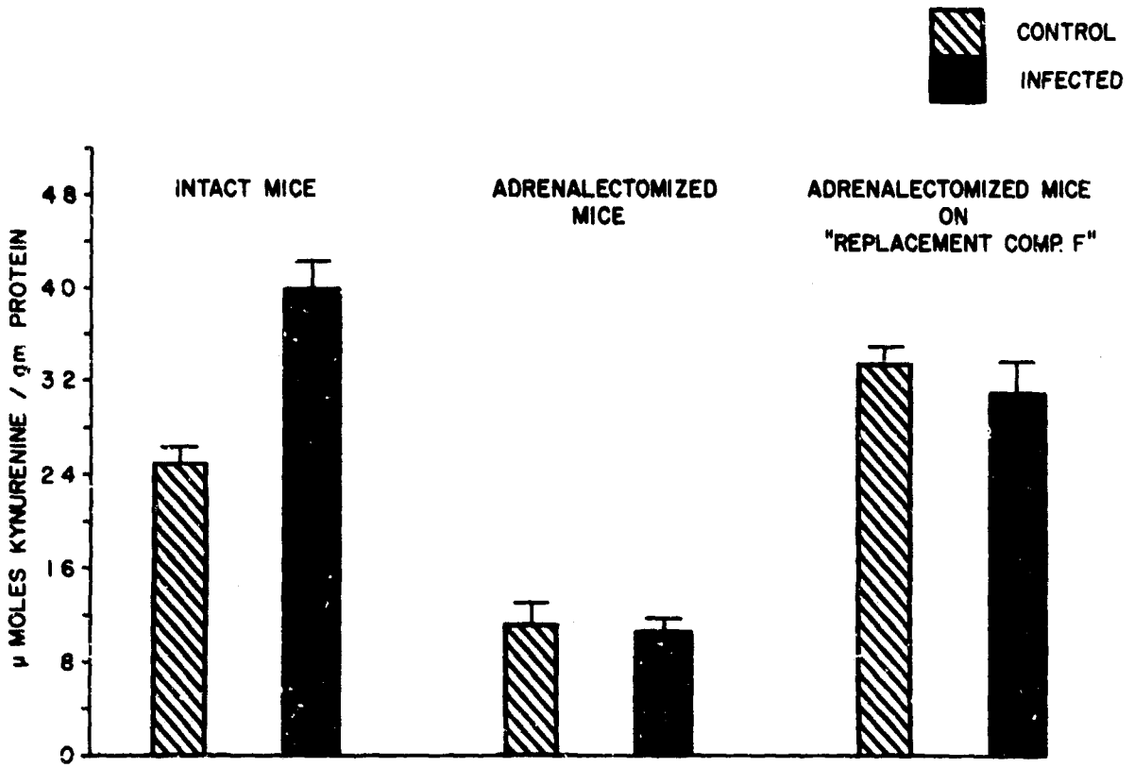

FIGURE 4. INFLUENCE OF PNEUMOCOCCAL INFECTION (SMALL INOCULUM) ON TRYPTOPHAN PYRROLASE ACTIVITY 2 HOURS POSTINOCULATION.

Determinations of enzyme activity in the terminal stages of infection as shown in earlier figures have indicated that values are normal or only slightly above controls. This observation seemed somewhat incongruous since the terminal stages of the infectious process were associated with a maximal endogenous adrenal response and presumably one would predict similarly elevated values for tryptophan pyrrolase. Therefore a series of studies were performed to define the responsiveness of the tryptophan pyrrolase induction mechanism at different times of infection. As indicated earlier exogenous steroids produce 2- to 3-fold increases in enzyme activity within 3-4 hr of administration. In Figure 5 are shown 3 series of experiments to test the hormonal influence of tryptophan pyrrolase synthesis in infection. Mice given cortisol 3 hr prior to sacrifice are shown in black. To the left are shown noninfected controls inoculated with sterile media 6 hr prior to sacrifice. It may be seen that cortisol administration produced almost a 2-fold increase in enzyme activity in the noninfected mice. In the middle are shown mice infected with the small inoculum of pneumococci 6 hr prior to sacrifice. These infected mice had higher than normal baseline values but responded significantly after cortisol induction. Finally the effect of the large inoculum of pneumococci on inducibility is shown on the right. In spite of a lower than normal baseline value, the administration of cortisol was accompanied by a significant increase in enzyme activity. From these data it is clear that despite variation in the baseline value of enzyme activity during the early period of infection, hepatic tryptophan pyrrolase is still responsive to induction by a pharmacologic dose of cortisol.

In contrast, results of a similar series of experiments performed after 15 hr of infection are shown in Figure 6. It may be seen that mice infected with the large inoculum of pneumococci are no longer responsive to cortisol induction as shown on the right; but those mice infected with the small inoculum retain a portion of their initial responsiveness as shown in the central pair. It may be concluded that infection with a large inoculum of pneumococci imposes, by 15 hr, a pronounced inhibitory influence on new enzyme synthesis.

Figure 7 shows another series of experiments identical to the previous figures with one exception, all mice were sacrificed after 21 hr of infection. Despite the late increase above control values for tryptophan pyrrolase during the terminal period in infected mice, responsiveness to cortisol induction is inhibited with both large and small inocula. These experiments suggest that as infection progresses factors not yet identified appear which are inhibitory to induction of tryptophan pyrrolase even by pharmacologic amounts of cortisol.

Figure 8 shows a series of experiments which were done to obtain more information relative to this late inhibitory influence. These studies were performed in adrenalectomized mice given replacement cortisol. It may be seen that these adrenalectomized mice receiving a fixed steroid replacement schedule have a progressive reduction in enzyme activity which is significant after 26 hr of infection.

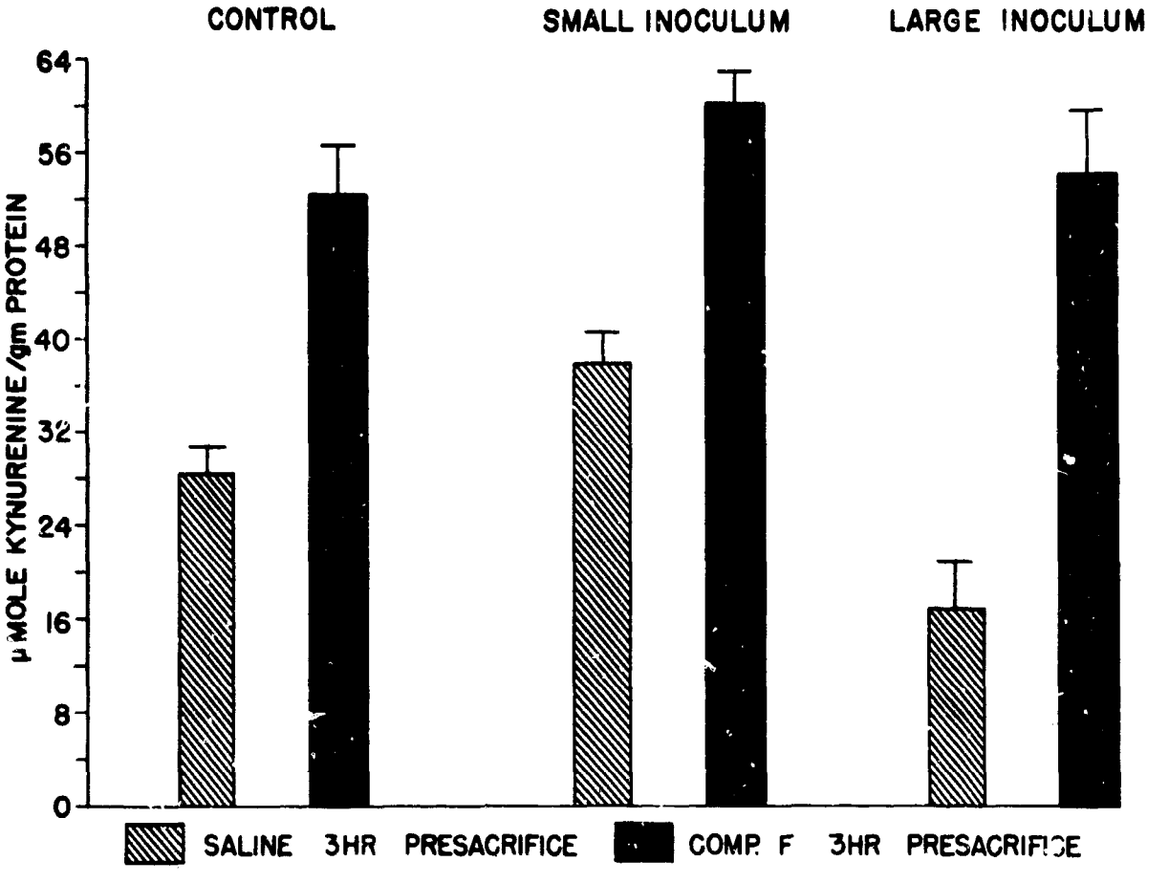

FIGURE 6. INFECTION-PRODUCED MODIFICATION OF HORMONAL INDUCTION OF TRYPTOPHAN PYRROLASE 15 HOURS POSTINOCULATION.

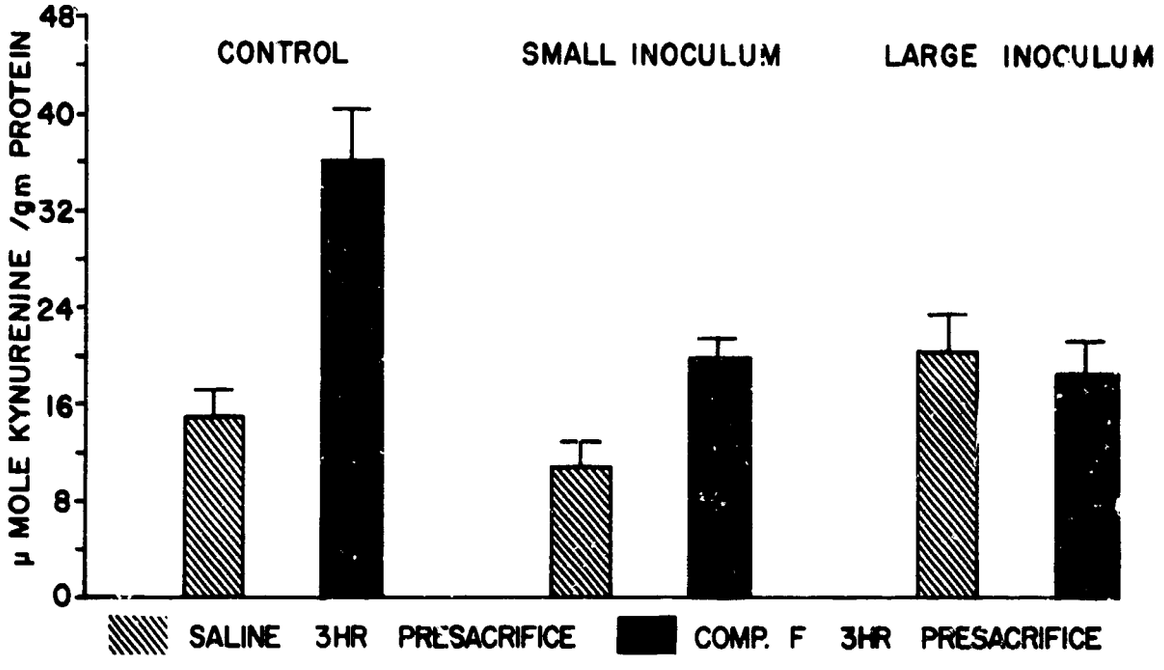

FIGURE 7. INFECTION-PRODUCED MODIFICATION OF HORMONAL INDUCTION OF TRYPTOPHAN PYRROLASE 21 HOURS POSTINOCULATION.

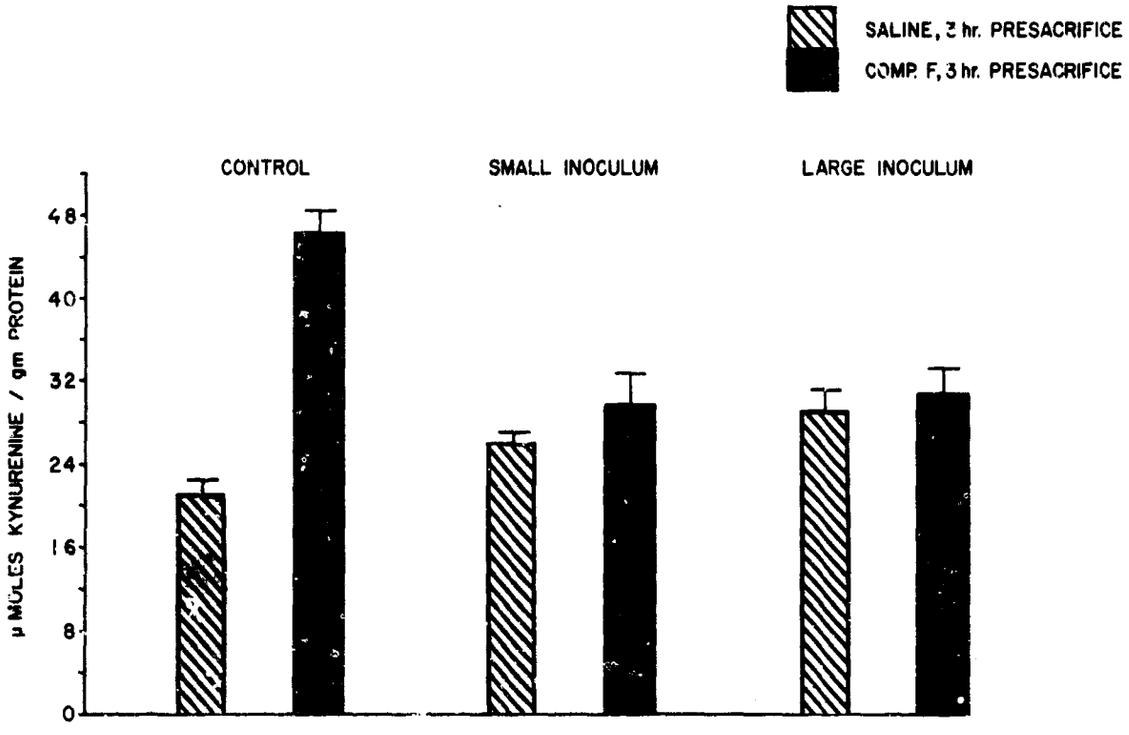

FIGURE 5. INFECTION-PRODUCED MODIFICATION OF HORMONAL INDUCTION OF TRYPTOPHAN PYRROLASE 6 HOURS POSTINOCULATION.

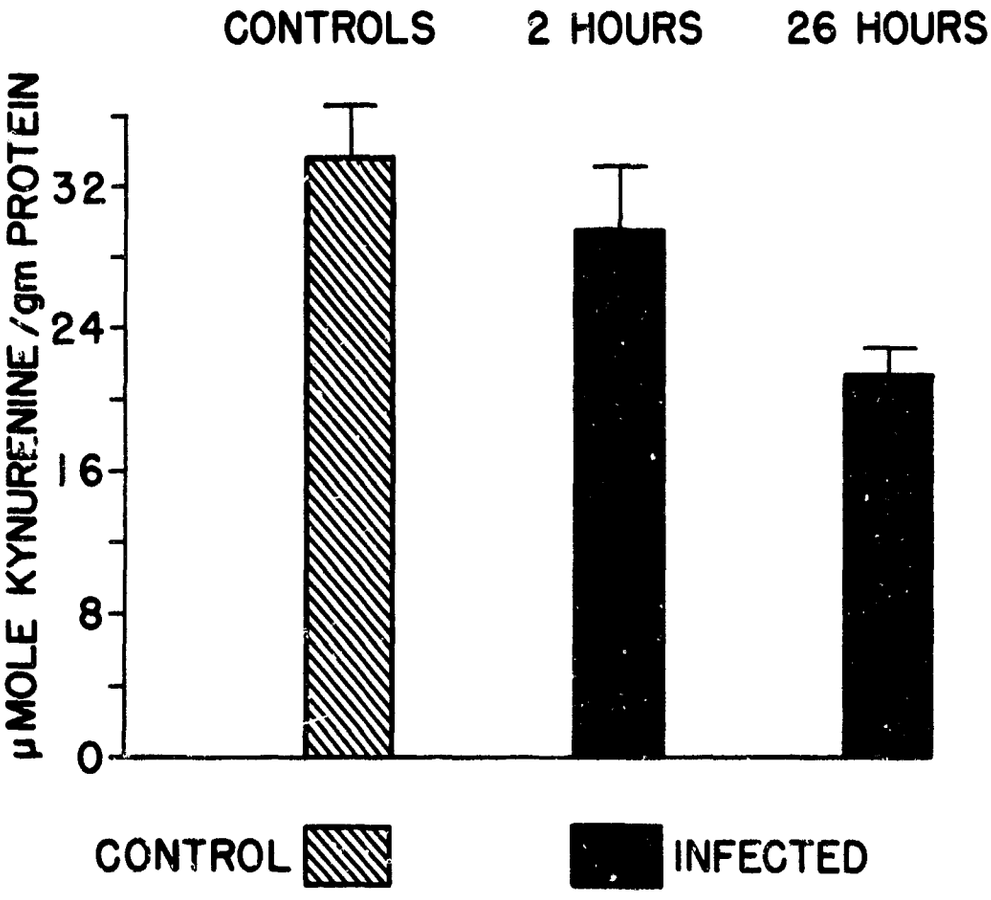

FIGURE 8 INFLUENCE OF PNEUMOCOCCAL INFECTION (SMALL INOCULUM) ON TRYPTOPHAN PYRROLASE ACTIVITY IN ADRENALECTOMIZED MICE ON REPLACEMENT COMPOUND F

The data presented herein allow us several conclusions. First, serial determinations of tryptophan pyrrolase and perhaps other enzymes must be interpreted with consideration given to circadian variation. Second, it is apparent that during acute infection there are both stimulatory and inhibitory influences on tryptophan pyrrolase activity. The nature of these influences and for that matter the number that exist are not at all clear. For this reason simple static measurements of the enzyme tryptophan pyrrolase must be interpreted as a summation of a variety of simultaneous and possibly divergent influences. Increases and decreases in activity may reflect changes in rate of enzyme synthesis and/or degradation, both of which alter actual enzyme population. Changes in cofactors and coenzymes would be expected to alter enzyme activity without actually changing enzyme amount. The third conclusion that appears warranted is that the early increase in enzyme activity noted after inoculation with a small number of organisms requires an intact adrenocortical function. Data is insufficient to state whether this increase reflects new enzyme synthesis, reduction in degradation rate, removal of an inhibitor, or a combination of these or other factors. This aspect is being studied in more detail at this time. The fourth conclusion that appears warranted from our data is that well established inhibitory influences to cortisol induction of tryptophan pyrrolase appear somewhat later in infection. It is apparent that pharmacologic doses of steroid are not effective in overcoming this inhibitory influence. The fact that enzyme activity in the terminal stages of infection is normal or slightly above normal must be interpreted in the context of a maximal adrenal response, a state that would be expected to produce marked elevations in tryptophan pyrrolase activity. The nature of the inhibitory process or processes is not clear from our data. The existence of a repressor factor has been postulated by Garren et al[10] and it is conceivable that generalized pneumococcal infection activates a repressor system. Growth hormone which also blocks tryptophan pyrrolase synthesis could play an inhibitory role in infection. Unfortunately nothing is known regarding the secretion of growth hormone and its activity in infection. Also to be considered is the mere presence of proliferating microorganisms within the hepatic tissue. However the inhibition of tryptophan pyrrolase induction occurs prior to the appearance of large numbers of microorganisms within the liver.

SUMMARY

Both stimulatory and inhibitory influences have been observed to be at work on the tryptophan pyrrolase induction system. Adrenal steroids play a major role in the stimulatory aspects which we have noted. Data are not available as yet to state categorically whether changes in tryptophan pyrrolase activity in pneumococcus infection actually represent changes in enzyme synthesis or indirect changes in cofactor or substrate concentrations. It is apparent that additional studies will be required to define better the nature of the factors that mediate early host adaptive changes in infection.

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ALTERATIONS OF HOST CELLULAR RIBONUCLEIC ACID METABOLISM DURING INFECTION

George Lust, Ph.D.*

The experimental results to be described are an extension of our previous work concerning alterations in host protein metabolism. Our previous experiments were primarily concerned with investigating overall host protein synthesis during infection. The principle findings in this area were that during an arbovirus infection the rate of protein biosynthesis in mouse liver was markedly decreased early during the infection, but had returned to normal values before clinical symptoms could be observed.[1-3] Under somewhat more controlled conditions, the same trend was observed in mouse fibroblast cells (strain L) in tissue culture.[4] Protein synthesis was depressed very rapidly in these cells, but appeared near normal at the time mature virus was produced in the cells. In infected L-cells, we also demonstrated the presence of a new ribonucleic acid (RNA) polymerase, not present in normal cells, which is involved in the synthesis of new viral RNA.

A somewhat different pattern or protein synthesis was observed during bacterial infection. During Diplococcus pneumoniae infection the reverse situation was actually found, in that the rate of protein synthesis in liver was elevated over control levels.[2,3]

From this brief summary of earlier work, it is apparent that overall host protein synthesis is indeed altered during infection. Therefore, since infection influences protein synthesis, it may be hypothesized that one or more of the specific reaction sequences required for synthesis may be affected.

Since RNA species are known to play a central role in protein biosynthesis, it was of interest to study these cellular constituents more intensively during the stress of infection. The results and thoughts presented have been derived mainly from work utilizing the mouse liver system. We have also investigated other tissues such as brain, intestine and muscle, but liver always exhibited the most noticeable effects.

There are basically 4 different groups of RNA involved in the protein synthesis: messenger, transfer, a large ribosomal subunit, and a smaller ribosomal subunit. The messenger is transcribed in the nucleus of cells and then passes into the cytoplasm, where it functions in assembling the ribosomes into a functional polyribosome needed for protein synthesis. The transfer, or soluble, RNA brings the activated amino acids to the polyribosome to be incorporated into proteins. Also required are transfer and releasing factors, as well as enzymes to assemble the polypeptide chain. From the literature, it is known that ribosomal RNA is more stable (slower

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turnover time) than messenger RNA.[5] As we shall see, the changes that we have observed are in large part attributable to alterations in messenger RNA. This report is concerned with studies on the factors which may alter host RNA species quantitatively.

Male white mice (CD-1) were infected by injecting subcutaneously in the back approximately 10 organisms of D. pneumoniae, type $1A_5$ suspended in tryptose-phosphate broth. The mice died within 72 hr. Suspending medium without organisms was injected into controls.

The mice used in the experiments weighed between 25-35 gm. Livers of 10-12 mice were pooled, minced and homogenized, and the desired subcellular components were obtained by differential centrifugation. Microsomes and pH 5 enzymes (soluble enzymes) were obtained from livers of both control and infected animals and frozen at -20 C until all samples had been collected. Incubations were performed, protein and radioactivity quantitated, and the results were expressed as counts/min per mg protein.

In vitro microsomal protein synthesis experiments were conducted using suitable, standard incubation conditions with C^{14}-leucine as the tracer, and the microsomes and soluble enzyme systems of livers. The soluble enzymes (pH 5) contained both activating enzymes and soluble RNA. Two kinds of experiments were performed: (1) microsomes, obtained from infected livers and uninfected control livers, were incubated with their respective soluble enzyme systems (pH 5 enzymes), (2) the microsomes, isolated from control livers and from livers of pneumoniae-infected mice, were incubated with the pH 5 enzymes from uninfected control livers only. The data of these experiments are presented in Figure 1.

Part A represents the microsomes incubated with their own respective cellular pH 5 enzymes. In Part B all microsomes were incubated with control, uninfected pH 5 enzymes only. The data are shown with the range of the individual determinations. In both experiments an increase of in vitro C^{14}-leucine incorporation into protein was observed. When the microsomes were incubated with their respective pH 5 enzymes, protein synthesis increased about 35% during the infection. When pH 5 enzymes from uninfected livers were used with microsomes from infected livers, protein synthesis was elevated about 40% (Part B, Figure 1) above control levels. In both experiments the rate of incorporation dropped rather sharply as the mice became moribund and were close to death. From these experiments, it was apparent that the increase in protein synthesis in the microsomal fraction seen at 16 and 40 hr postinjection of the pathogen could be accounted for by an increased activity in the microsomal fraction itself, since the soluble cellular fraction apparently exerted no unusual influence. This indicated that the transfer RNA was not altered during this infection.

In order to try to account for the microsomal increase, sucrose density gradients were employed. The sucrose density gradients indicated that during the bacterial infection the quantity of liver polyribosomes

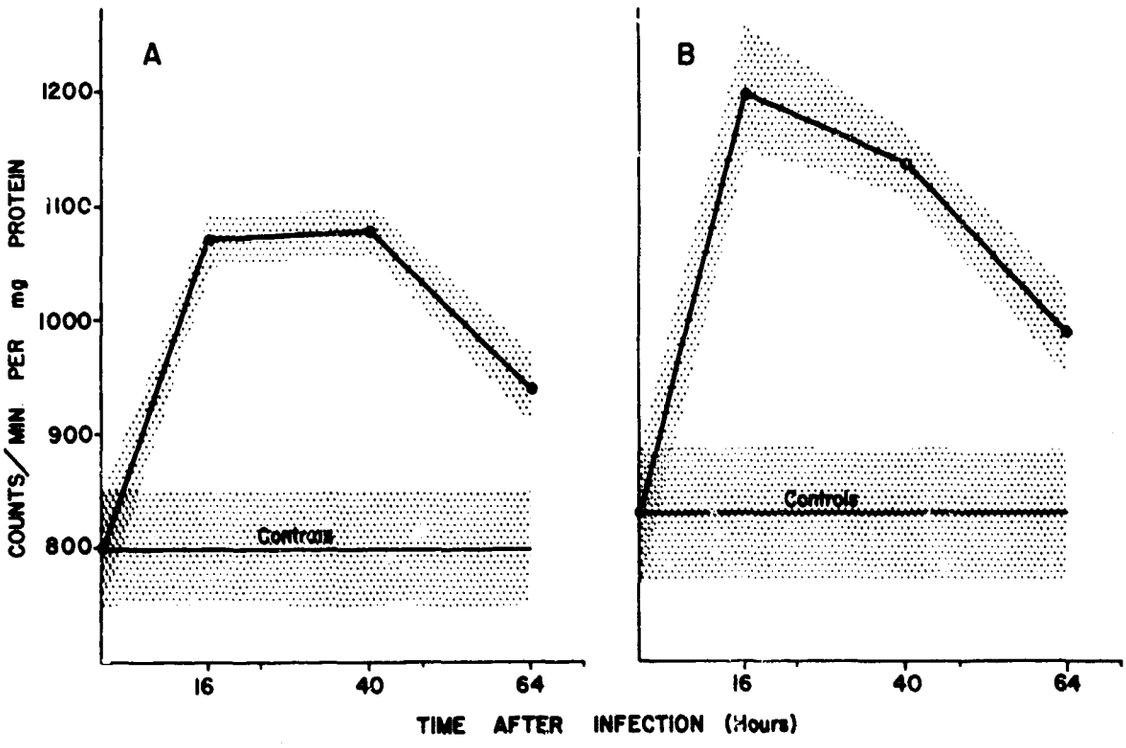

FIGURE 1. MICROSOMAL PROTEIN SYNTHESIS IN VITRO DURING BACTERIAL INFECTION.

may be greater than in uninfected livers (Figure 2). This figure presents results from 15-34% linear sucrose gradients. Linearity was checked by measuring the index of refraction of each fraction (dashed line). Readings of ribosome distribution were made at 260 mμ in a spectrophotometer. The line representing the control ribosome pattern (circles) has a large peak between fractions 10 and 16 and a smaller peak in fractions 17 to 21. The latter peak represents monosomes and disomes. This was ascertained by treating the control sample with a definite quantity of ribonuclease at 0 C for 30 min (dotted line). This procedure destroys messenger RNA dispersing the polymer into monosomes-disomes. The line with triangles represents the pattern seen in a preparation from infected animals. It can be seen that in livers of mice infected 18 hr monosomes and disomes are no longer to be found. Under these conditions, it was characteristic that a monosome-disome peak was present in uninfected control livers and absent during the early phase of infection. Intraperitoneal (IP) administration of hydrocortisone (3 mg) to uninfected mice also caused the disappearance of the monosome peak in liver preparations. These changes may be attributed to increased messenger RNA synthesis.

In support of this hypothesis, we have found an increased rate of uridine-2-C^{14} incorporation into RNA in liver during this bacterial infection (Figure 3). In this case uridine-2-C^{14} was injected into the tail veins of mice; the radioactivity in microsomal RNA of liver was quantitated. It is readily apparent that the infected animals exhibited an increased rate of RNA synthesis.

All these data indicated that increased messenger RNA synthesis may be responsible for the increased liver protein synthesis. It is well known that hormones influence protein and RNA synthesis. Cortisone and cortisol have been shown to stimulate messenger RNA and protein synthesis very rapidly. For this reason, the correlation of the response to infection and hormone administration was investigated. Adrenalectomized mice were used for this study. Adrenalectomized mice were treated IP every 12 hr with saline or 0.1 mg or 3 mg cortisol/mouse (Figure 4). C^{14}-leucine was injected into groups of control animals and infected animals, and the microsomes isolated as before. First, it is evident that with increasing concentrations of cortisol, protein synthesis also increases. It is also clear that adrenalectomized mice do not respond to this bacterial infection by increasing liver protein synthesis. The control values remained unchanged in infected animals. Therefore, it appeared that adrenal hormones play an important role in liver RNA synthesis during infection. The primary host response which activates the pituitary-adrenal axis is at present not well defined.

The factors influencing host cellular metabolism during a virus infection are apparently more complex. A generalized stress response to the infection may limited, as in the case of the bacterial infection; however, the infecting agent also interferes directly with cell metabolism at a very basic level.

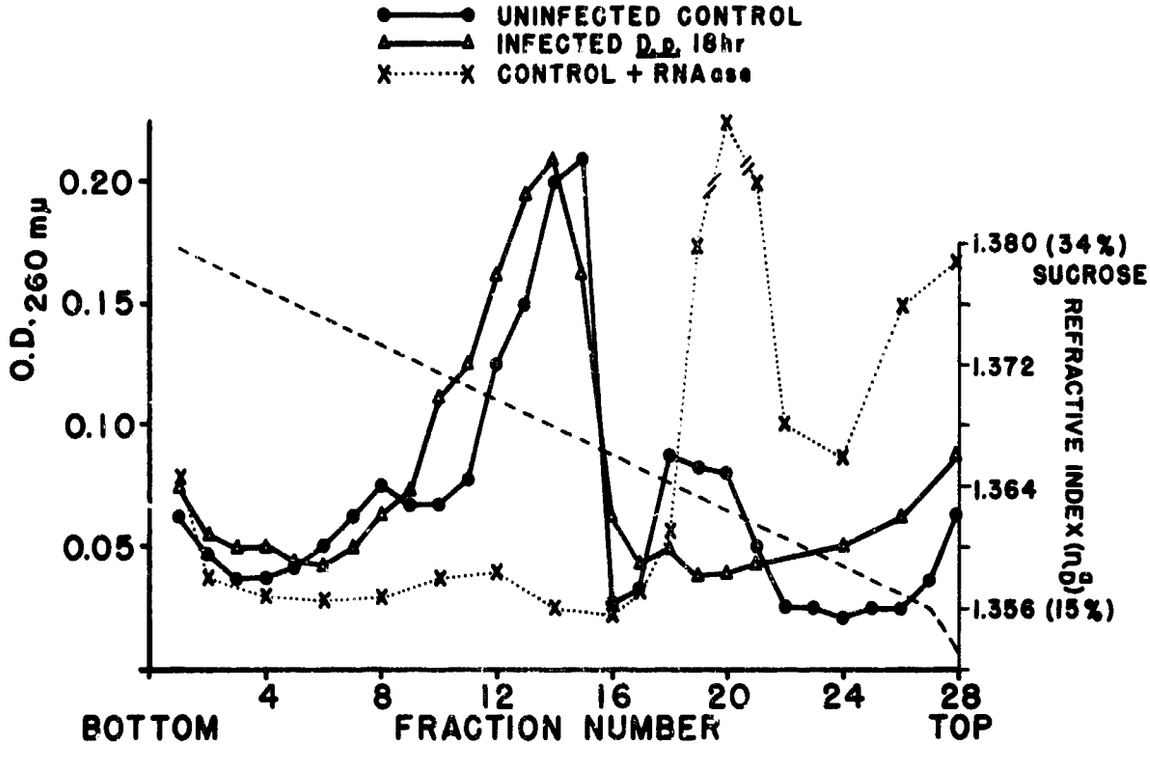

FIGURE 2. SUCROSE-DENSITY GRADIENT STUDY OF MOUSE LIVER RIBOSOMES IN BACTERIAL INFECTION.

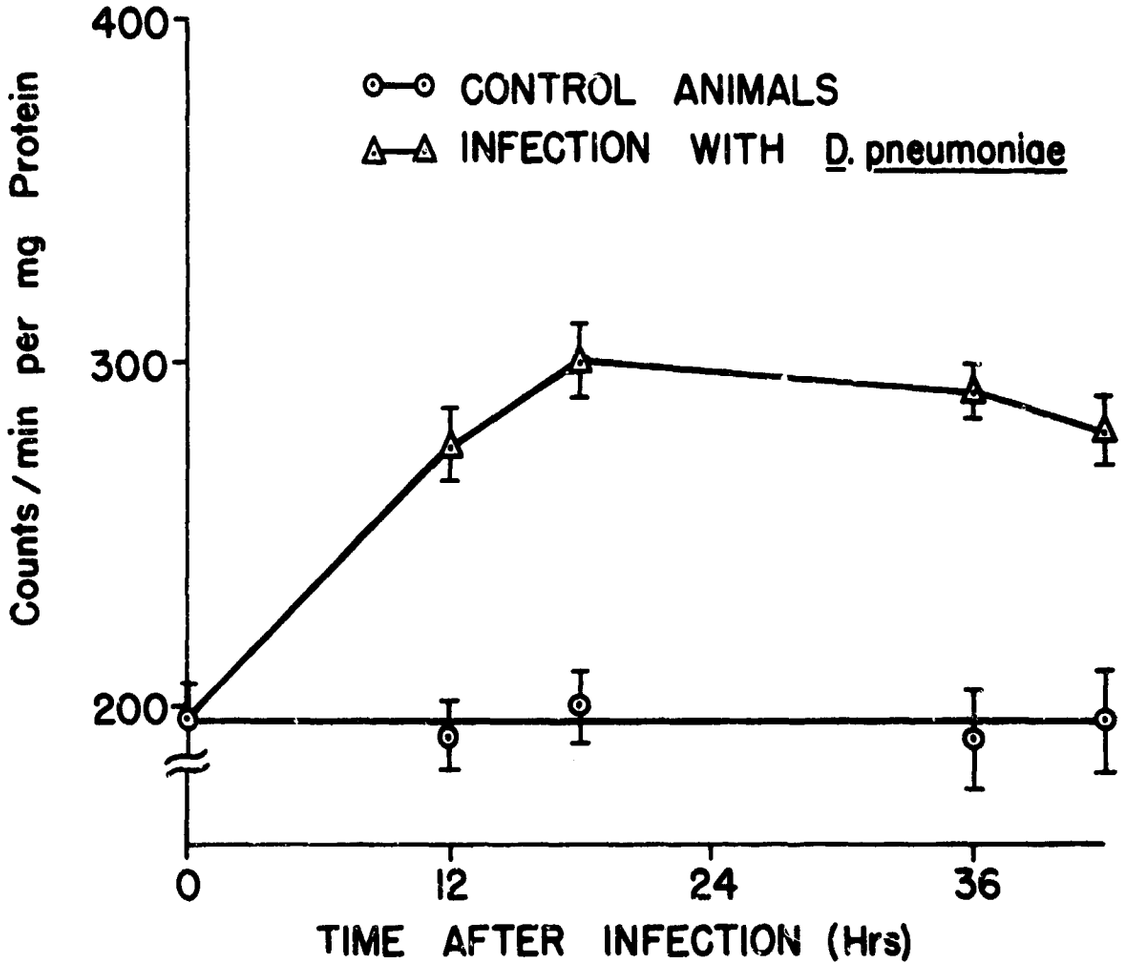

FIGURE 3. INCORPORATION OF URIDINE-2-C^{14} INTO MOUSE LIVER MICROSOMAL RNA

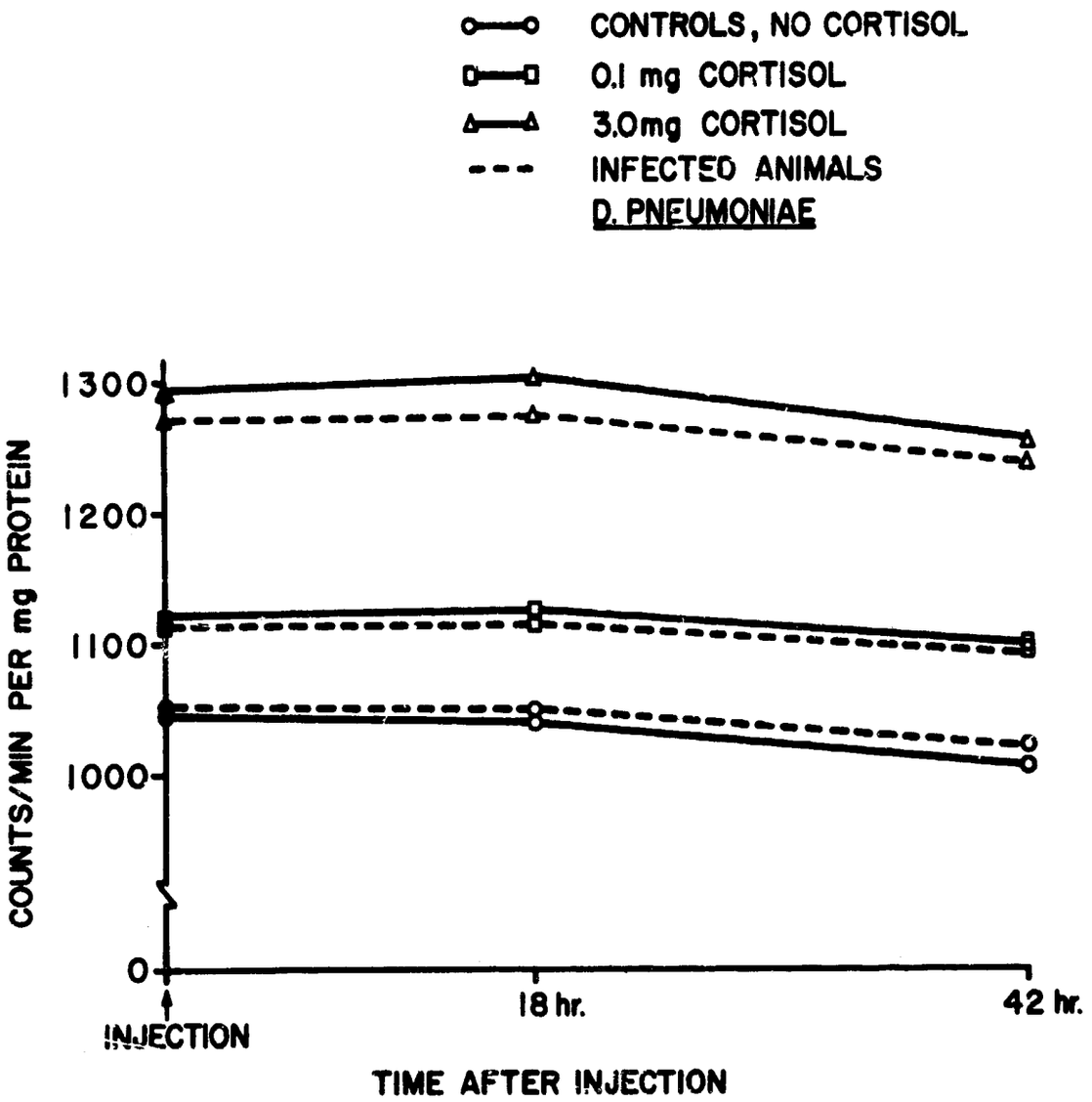

FIGURE 4. MICROSOMAL PROTEIN SYNTHESIS IN VIVO IN ADRENALECTOMIZED MICE.

For our model system we used the Trinidad strain of Venezuelan equine encephalomyelitis (VEE) virus to infect mice. In tissue culture this virus replicates in the same manner as do picorna viruses. Mice were infected by injecting IF 3000-4000 median lethal doses of VEE virus. In vitro protein synthesis was investigated with the microsomal and soluble fractions from livers of virus-infected and control mice.

The results summarized in Figure 5 are again presented as cpm/mg protein. It was found that protein synthesis was decreased during the early period of infection. The rate of synthesis was depressed 1 and 2 days after inoculation, but returned to normal levels after 4 days. The decrease varied between 17 and 36% below uninfected control values. When microsomes were incubated with their respective pH 5 enzymes, the decrease was about 30% (on the left). However, the decrease was only 17% when control pH 5 enzymes only were used (on the right). These results indicated that both the microsomal fraction as well as the soluble enzyme fraction may have been adversely affected during the virus infection.

To study this phenomenon further, we once more turned to sucrose density gradients. Penman et al[5] had reported that a breakdown of polyribosomes occurred in poliovirus-infected HeLa cells. A sucrose gradient study was therefore made of mouse liver ribosomes during VEE infection. This is illustrated in Figure 6. All conditions were the same as for the earlier gradient analysis. The dashed line represents the uninfected control group. The large polyribosome peak was again evident, as was a small peak for monosomes. On the other hand, in the livers of infected animals, the polyribosomal peak was missing. Also, the monosome-disome peak in this instance was very much larger than in normals. This did indeed indicate a disruption of the polyribosomes. The exact mechanism responsible for this phenomenon is not clear as yet. However, we[4] and others[7] have reported that during arbovirus infection host RNA synthesis is greatly reduced early in the infectious cycle.

Another interesting finding in this gradient study was the appearance of a very heavy aggregate, found toward the bottom of the tube. We have as yet done no infectivity assays on these fractions, but this peak may represent an aggregation of mature virus particles.

It is clear that host ribonucleic acid metabolism is altered in mouse liver during infections. By incorporating uridine-C^{14} into RNA, it was seen that an increase in synthetic rate occurred during D. pneumoniae infection, and a decrease during the arbovirus infection. Similar conclusions were drawn indirectly from the in vitro protein synthesis experiments, and the sucrose gradient studies.

It was of interest to study RNA directly by isolating it from the rest of the cellular components in relatively pure form. We have done this. I think this phase of the work may provide some very exciting answers to current problems.

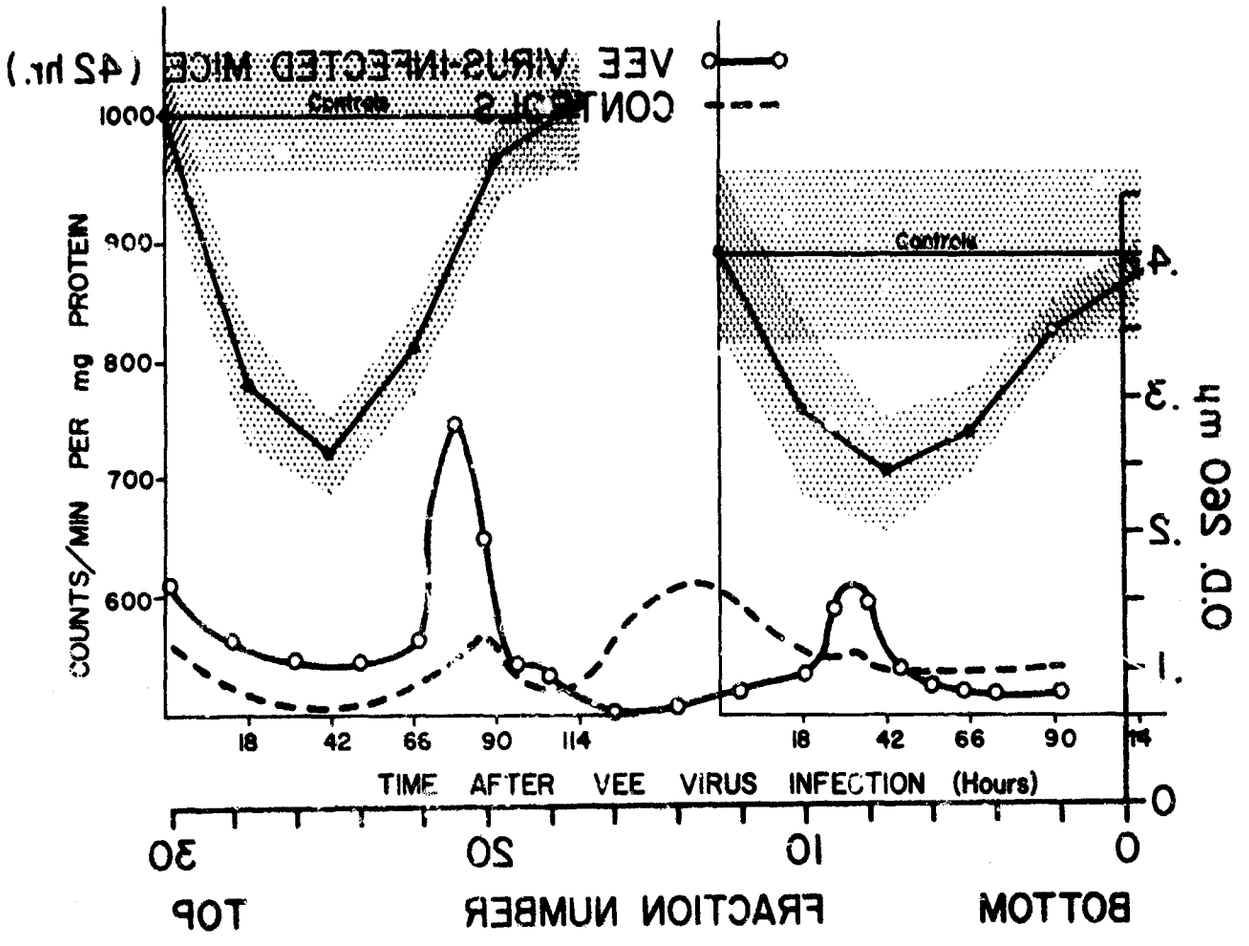

FIGURE 5. LIVER MICROSOMAL PROTEIN SYNTHESIS IN VITRO DURING VIRUS INFECTION.

FIGURE 6. SUCROSE DENSITY GRADIENT STUDY OF MOUSE LIVER RIBOSOMES IN VIRUS INFECTION

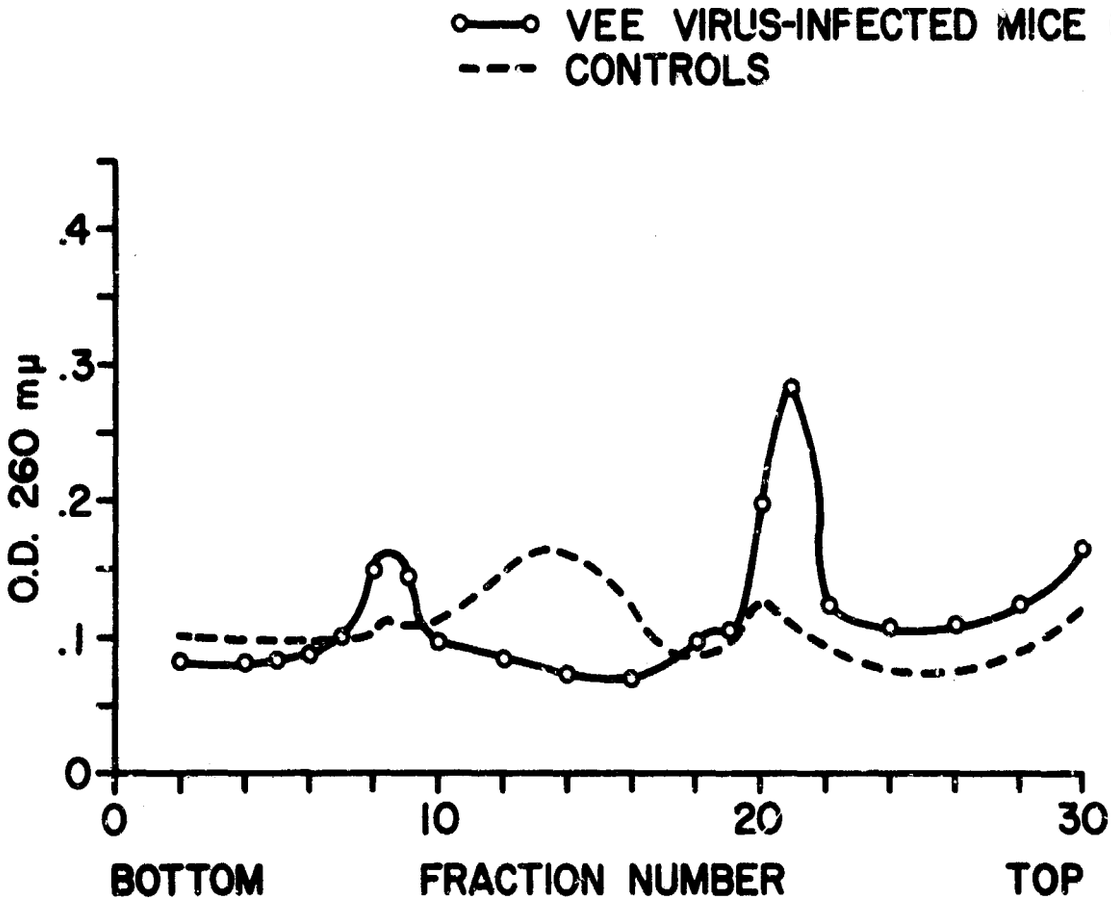

FIGURE 6. SUCROSE DENSITY GRADIENT STUDY OF MOUSE LIVER RIBOSOMES IN VIRUS INFECTION

Infection may elicit an adrenal hormone response. The hormone interacts at the gene level with deoxyribonucleic acid (DNA), causing increased messenger RNA synthesis, and subsequently increased protein formation. This is the pattern seen in liver. It is not a universal phenomenon; contrary to this, in muscle, cortisol is known to decrease protein synthesis. We have also obtained data to support this concept.[2,3] The RNA-virus also alters metabolism more specifically. First, it is known to inhibit DNA directed RNA synthesis. This means it is preventing host messenger RNA formation. Secondly, since the viral RNA is the template for new viral RNA formation and new enzymes are induced, the viral RNA may also serve as messenger RNA. At any rate, it becomes clear that host messenger RNA should appear in lesser amounts in the polyribosomes during virus replication. We would like to be able to measure these alterations quantitatively; it is this very problem that will be described.

The methods in general use for fractionating RNA are the sucrose density gradient and also the methylated albumin or Kieselguhr column. These methods are used extensively, do not have a very high degree of resolution, are laborious, and take relatively long periods of time to perform.

Recently I had the opportunity to spend some time in the physiological chemistry laboratory of H. N. Munroe at the Massachusetts Institute of Technology, Cambridge. Dr. Munro[8] allowed me to become acquainted with his very promising and as yet unpublished method for separating RNA's. Basically the method involves electrophoresis of the isolated RNA on agarose gels at pH 7.9. The RNA components are rapidly and discretely separated from one another with a high degree of resolution. RNA's are seen in addition to those usually observed in a sucrose gradient. RNA can be isolated very satisfactorily by the phenol method from: whole homogenate, nuclei, microsomes, ribosomes, ribosomal subunits and from the soluble cellular fraction. This isolation was done; the isolate were then characterized further by both sucrose gradients and agarose gel electrophoresis.

Figure 7 illustrates the pattern of RNA distribution on a 5-20% sucrose gradient. When purified soluble RNA was layered on the gradient a peak appeared in this area with a sedimentation value of 4S; when ribosomal RNA was used 3 peaks were generally seen; a 28S and 18S peak corresponding to the 2 ribosomal subunits and a smaller RNA peak which is associated with the ribosomes; the material in the small peak is somewhat heavier than soluble RNA and is designated 6S in this case. We now know that this small peak in the gradient always contains impurities of 4S (soluble RNA) under our conditions. The peak under the dotted line, designated 36S, is the peak we expect to find for purified arbovirus RNA. We have a small stock of purified VEE RNA and Sindbis virus RNA. Based on the work of others, Sindbis virus RNA should give a peak in the same area.

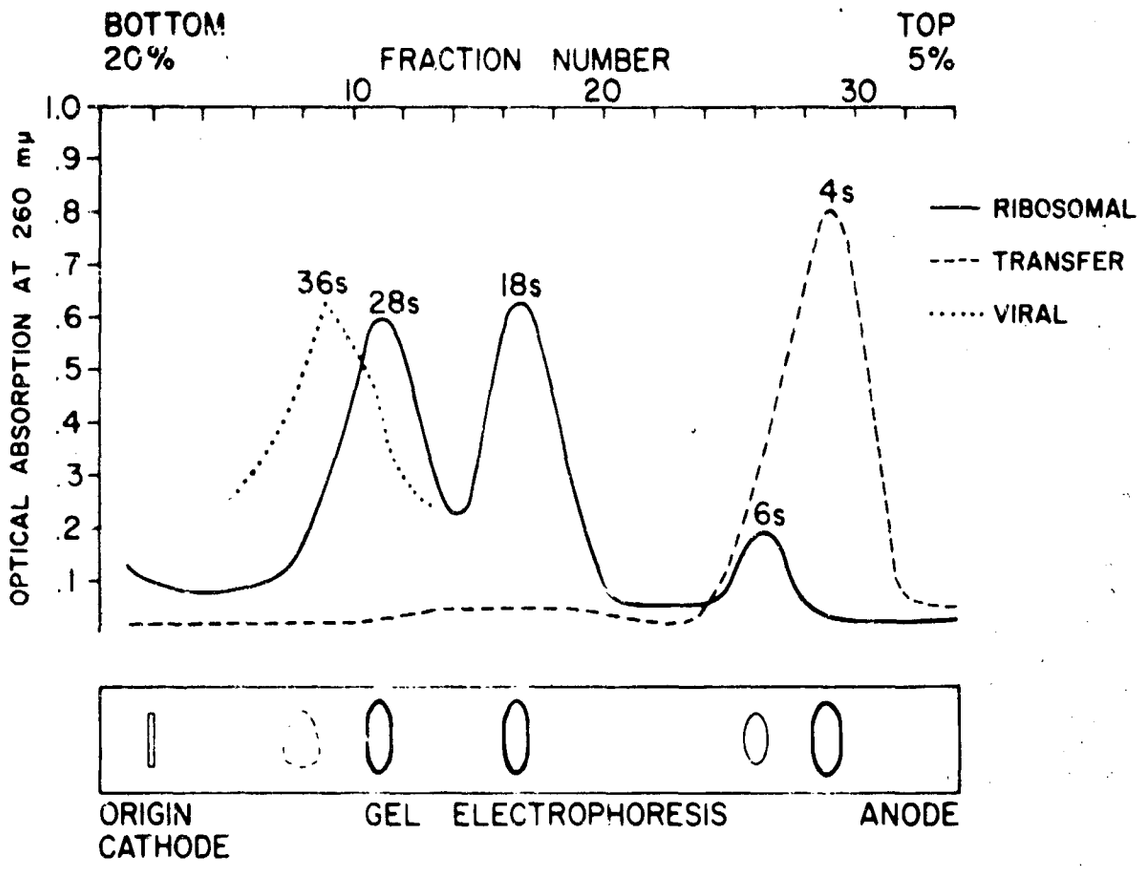

FIGURE 7. SUCROSE GRADIENT ANALYSIS OF MOUSE LIVER CYTOPLASMIC RNA.

At the bottom of this figure is a gel electrophoresis pattern of RNA moieties. The expected viral band should be closest to the origin, then the ribosomal subunit peaks, and much farther away 6S and 4S bands; the latter bands are clearly separated from each other on the agarose gel. As mentioned earlier we see RNA bands on the gel that cannot be observed with sucrose gradients; these were left out for clarity.

Figure 8 shows the pattern of cytoplasmic RNA species seen after agarose gel electrophoresis. The isolated microsomal RNA gave column 1 with 5 additional bands, 2 between 28S and 18S and 3 after 18S. The 4S and 6S peaks are again distinctly separated. Soluble RNA is shown in column 2. The ribosomal RNA (column 3) gave essentially the same pattern, except that the 4S band (soluble) was decidedly smaller. The soluble RNA (4S) was homogeneous on electrophoresis and we used this 4S RNA to identify soluble RNA in other RNA preparations. Column 4 represents the area where we are expecting to find arbovirus RNA.

Just a word on methodology: after electrophoresis for 90 min at 400 V in the cold room, the gel is stained with either toluidine blue or pyronine Y in order to see the bands. The method is very sensitive: 0.5 μg RNA can be easily detected and measured. Quantitation is carried out scanning the stained-gel with a Joyce recording microdensitometer and the Canalco microdensitometer. The densitometer graphs a profile which is easily amenable to quantitation. We have also used P^{32} incorporation into RNA and have counted the radioactivity directly in the gel. In preliminary work in which we stimulated liver messenger RNA production with cortisol and used P^{32} incorporation, we found that the specific radioactivity in RNA was doubled in the cortisol-treated animals. A large majority of the increased radioactivity was found in the 6S band of the gel electrophoresis profile.

SUMMARY

Electrophoresis on agarose gels separates RNA species discretely with good resolution. The method provides a rapid, sensitive way of screening RNA preparations for complexity of individual samples or in comparison of them from different sources or experimental conditions. The 6S RNA and the other minor components are apparently associated with ribosomes. Their nature is still uncertain. The observation that the 6S RNA is associated with the ribosomes may be of value in elucidating its function. It may well be that this band is where messenger RNA is found.

Although this method must still be refined in several ways, it is already very useful. Knowledge of the metabolic activity of the various RNA components will be of value in order to explain alterations that have been observed during infections. An emphasis on alterations of specific RNA molecules as well as other metabolites, should provide significant contributions in order to define the host metabolic response to infections more precisely.

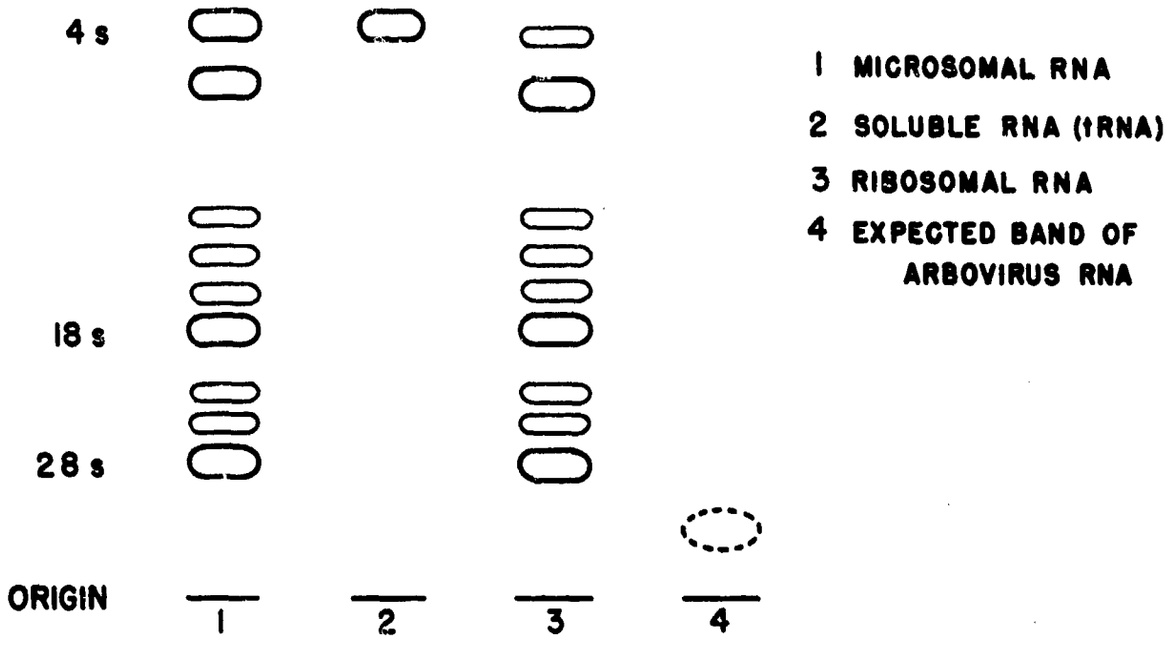

FIGURE 8. GEL ELECTROPHORESIS PATTERNS OF CYTOPLASMIC RNA SPECIES.

ACKNOWLEDGEMENT

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MECHANISMS OF ENDOTOXIN TOLERANCE*

Sheldon E. Greisman, M.D., Edward J. Young, and William E. Woodward, M.D.**

The present studies are a continuation of a series of investigations designed to explore the importance of bacterial endotoxins in the pathogenesis of Gram-negative bacterial infections in man. It has long been recognized that when a constant dose of endotoxin is administered to normal rabbits or to healthy volunteers as a single intravenous (IV) injection at 24-hr intervals, approximately 1 week is required for induction of maximum pyrogenic tolerance. In an earlier report[1] to the Commission, it was demonstrated that when bacterial endotoxins were infused continuously at a constant rate, maximum febrile unresponsiveness could be achieved within hours; moreover, in man, the subjective toxic reactions waned rapidly in parallel with the febrile response. To distinguish such acute unresponsiveness to endotoxin from the tolerant state induced by repeated daily single IV injections of this molecule, the term "pyrogenic refractory rate" was employed. Certain of the mechanisms underlying this pyrogenic refractory state have been explored in the earlier studies and it was inferred that (1) refractoriness resulted from inability of the host to continue to mobilize endogenous pyrogen during sustained endotoxemia, and (2) this in turn was consistent with specific cellular desensitization to endotoxin. The role of nonspecific mechanisms, however, including generalized depletion of "available" endogenous pyrogen, could not be excluded. The present studies were designed to explore further the concept that the pyrogenic refractory state is mediated by specific desensitization to endotoxin at the cellular level.

Healthy New Zealand albino rabbits weighing approximately 2 kg were obtained from a uniform source and acclimatized for pyrogen studies in fiberglass stalls. Following induction of the pyrogenic refractory state by a standard rate of IV Escherichia coli endotoxin infusion (Difco preparation 0127:B8) at a level of 18×10^{-4} µg/min, the pyrogenic responsiveness to a single IV test bolus of E. coli endotoxin was found to be significantly depressed. Indeed, the mean febrile response was reduced to levels observed in normal control animals receiving 1/50 this test dose. In contrast, rabbits that were rendered comparably refractory to the continuous infusion exhibited no reduction in pyrogenic responsiveness to a single IV bolus of preformed endogenous pyrogen or to agents known to liberate endogenous pyrogen, i.e. influenza virus (PR 8 strain), old tuberculin in specifically sensitized animals, or staphylococcal enterotoxin B (prepared by Dr. Edward J. Schantz and co-workers and supplied by the U. S. Army Medical Unit, Fort Detrick, Maryland). It is emphasized that the dosages of all test pyrogens

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were carefully selected to evoke febrile responses within the sensitive portions of their respective dose-response regions. In addition, continuous IV infusions of heat-killed pneumococcal suspensions resulted in maintained febrile responses, observations comparable to those of Atkins and Freedman[2] employing autoclaved staphylococci. Such responses presumably are mediated by sustained mobilization of endogenous pyrogen. Finally, it was observed that: (1) a continuous infusion of old tuberculin into specifically sensitized rabbits resulted in a pyrogenic refractory response pattern generally similar to that evoked by endotoxin; (2) endotoxin refractory phase plasma and liver homogenates exhibited no enhanced capacity to inactivate E. coli endotoxin pyrogenicity; (3) splenectomized rabbits readily developed the endotoxin pyrogenic refractory state and exhibited diminished inflammatory responses to intradermal testing with endotoxin; and (4) administration of fresh whole blood from normal donors containing an average of 1.6×10^8 granulocytes failed to restore febrile responsiveness to the continuing E. coli endotoxin infusion.

The present observations, considered together with those of other investigators, support the hypothesis that pyrogenic unresponsiveness to endotoxin involves 2 distinct immunologic mechanisms. In terms of this hypothesis, the rapid reduction in febrile unresponsiveness to endotoxin is mediated by desensitization at the cellular level. With small doses of endotoxin such as those employed in the present studies (< 1 μg) this desensitization is primarily specific; with larger doses, nonspecific mechanisms are superimposed. So long as the subsequent doses of endotoxin are closely spaced or continuously infused, optimal conditions are provided for cellular desensitization and pyrogenic unresponsiveness to a given quantity of endotoxin can be induced rapidly and maintained without the requirement for antibody. However, as the interval between endotoxin challenge is lengthened, cellular desensitization wanes and tolerance becomes increasingly dependent upon those antibodies directed against the common toxophore groupings responsible for endotoxin pyrogenicity which assist the reticuloendothelial system in the clearance and destruction of this molecule.

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THYPHOID FEVER: PATHOGENESIS AND PREVENTION

Richard B. Hornick, M.D.*

The study of typhoid fever induced in inmate volunteers has entered a new phase as one major goal has been achieved. Quanitative prophylactic efficacy of several vaccine preparations has been determined by utilizing varying challenge doses of the Vi antigen-containing strain of Salmonella typhosa, Quailes strain. These results are reviewed in Table I. The incidence of disease appeared to be almost identical in those men vaccinated with L (phenolized vaccine) or K (acetonized vaccine) following challenge with 10^5 organisms. No evidence of vaccine-induced resistance demonstrable in the groups challenged with an ID_{50} (10^7) or greater doses of typhoid bacilli. Analysis of O, H, and Vi antibody titers at time of challenge of the controls and vaccinees failed to demonstrate any correlation between level of antibody and resistance to infection. This is especially true in the controls challenged with the ID_{50} dose (Table II). In fact more of those volunteers who had no demonstrable O, H, or Vi antibody titers failed to become ill suggesting that in the absence of circulating antibodies disease does not necessarily follow the challenge. Obviously other circulating antibodies may be present which are important in resistance and have not yet been measured. However, in these controls with negative O, H, and Vi titers such an antibody (or antibodies) must be nonspecific and certainly unrelated to vaccine administration. If the ingested organisms were unable to multiply in the gut and invade the mucosa because of bacterial interference or other local factors, this would appear to be a much superior defense mechanism than reliance on humoral antibodies.

We think it necessary to emphasize the lack of differentiation in vaccine immunity in the volunteer study because these results imply that antigenic makeup of the individual products may not be as important as other investigators have suggested. In the World Health Organization field trials[17] vaccine K was found to be superior to vaccine L in protecting adults. In children both vaccines were effective. This superiority was attributed to the preservation of Vi antigen by acetone in contrast to the degradation of this antigen by heat and phenol in vaccine L.

Evidence accumulated in recent volunteer studies tends to discredit the importance of Vi antigen in immunizing man or indeed as an essential offensive weapon of S. typhosa. First, volunteers immunized with purified Vi antigen have not shown enhanced protection at any of the challenge levels. Protection following the ID_{25} challenge was similar to that obtained with vaccines K and L (Table I). Furthermore, the titers of Vi antibodies were very similar whether K or L vaccine was administered to volunteers suggesting that ample Vi antigen survived the phenol treatment.

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TABLE I. INCIDENCE OF TYPHOID FEVER IN MAN IMMUNIZED WITH VACCINES K AND L CHALLENGED WITH VARYING DOSES OF S. TYPHOSA

VACCINE	NO. CASES TYPHOID/NO. CHALLENGED BY DOSE		
	10^9	10^7	10^5
K	2/3 (66%)	12/28 (43%)	4/43 (9%)
L	3/4 (75%)	13/24 (54%)	3/45 (7%)
Vi		10/14 (71%)	2/13 (2%)
Control	4/4 (100%)	15/30 (50%)	28/104 (27%)

TABLE II. EFFECT OF PREVIOUS TITER ON INCIDENCE OF DISEASE FOLLOWING CHALLENGE WITH AN ID_{25} OF S. TYPHOSA IN MEN IMMUNIZED WITH K AND L VACCINES

TITER	NO. WITH DISEASE/NO. CHALLENGED					
	O Antibody	Control	H Antibody	Control	Vi Antibody	Control
<7.5					0/1	3/15
7.5					0/6	2/8
10	1/2	20/56	1/4	19/38		
15					1/10	5/10
20	0/12	6/24	1/2	5/15		
30					3/14	1/5
40	4/26	2/11	1/7	2/10		
60					2/14	1/8
80	0/28	0/5	1/3	0/7		
120					1/18	1/4
160	2/11	0/2	0/4	1/15		
240					0/9	0/2
320	0/2		0/12	0/8		
480					0/4	
640	0/1		2/22	0,		
960					0/1	
1280			0/13			
2560			0/11			
5120			0/1			
10,240			1/3			
TOTALS	7/82	28/70	7/82	27/72	7/70	13/44
%	85	40	85	38	10	30

Second, it is conceivable that many strains responsible for disease are lacking in Vi antigen or contain very little. Recently 7-log doses of 2 classical non-Vi-containing nonmotile strains, Ty2-W and 0901, have been used to compare individual attack rates with that experienced following Quailes strain (Table III). There appears to be some difference between

TABLE III. VIRULENCE OF 1 X 10^7 S. TYPHOSA ORGANISMS FOR MAN

STRAIN	NO. WITH DISEASE[a]/TOTAL	
Quailes	16/32	50%
TY2-V	2/6	33%
TY2-W	4/19	21%
0901	6/20	30%

a. Required antibiotic therapy.

the Quailes strain and the 2 antigen-deficient strains. Virulence then is less without the Vi antigen but disease did occur in 21 and 30% of challenged volunteers. Insufficient experience with the parent Ty2-V strain prohibited statistical comparison but suggested that it was not more virulent than the Quailes in man. In the mouse there was a 2-log difference in virulence (LD_{50}) between Quailes and Ty2-V, the latter strain having the increased lethality.

The clinical responses in volunteers were not as severe with these 2 antigen-deficient strains as has been observed generally with the Quailes strain. Although the fever curves were typical, the physical findings were less striking and there were fewer subjective complaints. No relapses occurred in any of the 10 who were treated. The most striking difference in these experiments from previous studies was the lack of positive cultures. Blood cultures were positive in 4 of 10 men; negative blood cultures have been very unusual in those volunteers with disease caused by the Quailes strain. One additional volunteer had asymptomatic typhoid bacteremia with the Ty2-W strain. Stool cultures were also unrewarding. In the 10 with disease, again only 4 had positive cultures. In fact 4 of these 10 failed to have either positive stool or blood cultures. If it were not for the knowledge of the challenge employed and the occurrence of similar illnesses with positive cultures in other volunteers, these cases, studied by themselves would be instances of fever of unknown origin. They all had, however, the classic stepwise fall of fever typical of the response to effective chemotherapy and the important physical finding of lower abdominal tenderness to palpation with the associated sensation of gas-fluid filled loops of bowel being displaced under the hand. Serological studies have not been completed. Two of the 10 were treated with ampicillin with satisfactory responses; this again was in contrast to the therapeutic failures

with this drug in patients ill with Quailes strain. This suggested that certain strains of S. typhosa are more susceptibile to ampicillin than others and this fact may account for the contradictory reports on effectiveness of this drug. It should be emphasized that one could not predict from in vitro tests that ampicillin would be more useful against the 0901 strain than the Quailes; tube dilution studies gave similar results for both strains.

Antigenic analyses of the strains isolated from these patients are incomplete. However, Tully et al[2] have reported studies in chimpanzees utilizing the same strains and could not demonstrate in vivo acquisition of Vi antigen. However, this was not true for H antigen. Some 1,2-W isolates from blood and stools did become motile in these studies. We have noted that several isolates from men challenged with strain 0901 were motile.

Mr. Howard Semins, a second year medical student, conducted amino acid and sugar utilizations of isolates from the stools of these patients. He was able to find 4 strains that had acquired or lost the ability to ferment certain sugars. This appears to involve at least 2 enzyme systems in each instance. These alterations were attributes of a strain of E. coli (14948) used for comparison studies and suggested the transference of genetic material between the Quailes strain and nosocomial E. coli. These hybrids have stimulated our interest for use as possible oral vaccine strains. The rationale for this approach is the convincing animal studies of Formal and colleagues[3] using E. coli - Shigella hybrids as successful oral vaccines to prevent shigellosis in monkeys.

SUMMARY

Although the volunteer studies have shown typhoid vaccines to be effective against low dose challenge, the exact mechanism or mechanisms of this action are not apparent. Vi antigen did not appear to be as necessary for the virulence of typhoid bacilli in man as in mice and probably was not important for the immunization of man. Hybrid strains of Salmonella typhosa have been isolated from the stools of volunteers.

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STUDIES ON ROCKY MOUNTAIN SPOTTED FEVER

SEROLOGIC RESPONSE IN MAN TO VACCINATION WITH COMBINED EPIDEMIC TYPHUS, ROCKY MOUNTAIN SPOTTED FEVER AND Q FEVER VACCINE

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Simplification of vaccine administration by combining several monovalent vaccines into a single polyvalent product is desirable. Preliminary experiments were performed to compare the immunogenic potency of a single dose of composite epidemic typhus, Rocky Mountain spotted fever (RMSF) and Q fever vaccines with that of the component vaccines administered individually.

The vaccines employed were commercial products prepared by Lederle and Company (Epidemic Typhus Vaccine Lot 077-105, 1.0 ml and RMSF Vaccine Lot 065-152, 1.0 ml) and Walter Reed Army Institute of Research (Q Fever Vaccine Phase I, Henzerling strain Lot 1, 0.6 ml) for human use. The composite vaccine contained these in each 2.6 ml dose administered.

Complement fixing (CF) antigens employed in the testing of sera were supplied by the Department of Microbiology, University of Maryland School of Medicine: Epidemic Typhus Soluble Antigen, 13 July 1964, titer 1:64, Rickettsialpox Soluble Antigen, 17 May 1966, titer 1:32, and Q Fever Antigen, Nine Mile Strain, Phase II, Q75.

Since preliminary experiments in 7 men had shown that the combined vaccine was well tolerated,[1] 40 healthy informed male volunteers from the Maryland House of Correction were selected for comparative testing of the combined vaccine. These men were randomized into 4 groups of 10 men each; Group A received 2.6 ml of the combined vaccine, Group B, 1.0 ml of the component typhus vaccine, Group C, 1.0 ml of the RMSF vaccine and Group D, 0.6 ml of the Q Fever vaccine. Groups A, B and C received the vaccine subcutaneously (SC), one-half of the total dose being given into each deltoid whereas Group D received the entire dose of Q fever vaccine (0.6 ml) SC in the right deltoid. The men were examined daily for 10 days for signs or symptoms of local or generalized reaction to the vaccines; this included daily oral temperatures, examination of the vaccination sites and recording of subjective complaints.

Blood specimens were taken prior to vaccination and again 1, 2, 3, 4, 5, 6, 7, 9, 11, 13, 14, 18, 20, 22, 24 and 26 weeks after vaccination. These bloods were tested by CF for antibodies using standard microtiter techniques. All specimens were tested against each of the antigens on the same day. The lowest dilution of serum tested was 1:2. Titers were reported as the highest dilution giving 3+ or better fixation of complement.

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No significant system reactions were noted in any of the vaccinees. In only a few subjects did local reactions occur at the site of vaccination; they consisted of erythema and local edema, similar to that which might be seen following an injection of tetanus toxoid.

Of the ten men receiving monovalent typhus vaccine, 2 had evidence of antibodies (titers 1:4 or 8) in their baseline sera and both of these demonstrated rises in titer postvaccination. Three baseline specimens were anticomplementary which interfered with the interpretation of the low titer obtained in 2 of these men subsequent to receiving vaccine. Only 2 of the remaining 5 developed antibody in low titer (1:2) demonstrable in only a single specimen from each. Of the 10 men receiving combined rickettsial vaccine, one had a baseline titer of 1:2 and experienced no rise and another individual's specimens were all anticomplementary. Five of the remaining 8 developed CF titers of 1:2 to 1:8 against typhus antigen; 3 failed to demonstrate any antibody response.

Of those receiving monovalent RMSF vaccine, 8 gave evidence of an antibody rise although 3 had baseline titers of 1:2 or 4. Two failed to develop antibodies to RMSF antigen. Similar response to the RMSF component of the combined vaccine was shown: 6 of 9 men developed antibodies, 3 did not. All serum specimens from one subject in this group were anticomplementary.

All 10 men receiving monovalent Q fever vaccine developed antibodies in their serum at some time during the postvaccination period. Three of these men had low titers (1:2) in their baseline sera; one of these did not demonstrate a subsequent rise in titer. In contrast only 5 of 9 men acquired antibodies to the Q fever component in the combined vaccine; 2 of these had preexisting antibody. Four men remained negative throughout and again the specimens from one were anticomplementary. A summary is presented in Table I.

TABLE I. SUMMARY OF SEROLOGIC RESPONSES TO COMBINED RICKETTSIAL VACCINE (10 men per group)

CF ANTIBODY	COMBINED VACCINE GROUP			MONOVALENT VACCINE GROUPS		
	Antibody Rise	No Antibody Rise	?[a]	Antibody Rise	No Antibody Rise	?
Epidemic typhus	5	4	1	4	3	3
RMSF	6	3	1	8	2	0
Q fever	5	4	1	9	1	0

a. Indeterminate results. Baseline sera anticomplementary.

Antibodies were demonstrated where present 1 to 4 weeks after vaccine administration; in many their presence persisted for only a short time. Anamnestic response in those few men with preexisting antibody generally developed sooner and to higher titer. No remarkable differences were noted between response to combined vaccine as compared with monovalent vaccine, but the numbers are too small to detect subtle differences between groups. The volunteers still available for study have been given a booster dose of the respective vaccines and serologic testing is in progress. The antibody response to this second inoculation should be greater than that reported here after a single dose.

SUMMARY

A total of 17 men have now been given a combined epidemic typhus, RMSF and Q fever vaccine without untoward local or systemic effects. Preliminary serologic testing has revealed little if any difference in response to the components of this vaccine as compared with response to monovalent vaccine after a single inoculation. Additional studies are in progress to extend these observations and to measure the immunogenic effect of multiple dose vaccination.

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INFLUENCE OF TULAREMIA ON HUMAN INSULIN SECRETION*

George E. Shambaugh, III, Major, MC**

Carbohydrate metabolism is adversely influenced by infection. This observation first made over a hundred years ago in cholera patients has been reported many times during a variety of different infectious diseases.[1,2] The need for increased doses of insulin in the diabetic patient with superimposed infection is well recognized, but the mechanism underlying the progressive deterioration of previously adequate diabetic control which may begin before the onset of clinical illness remains obscure. Even the nondiabetic patient during a generalized infection has an increased fasting blood sugar and a deterioration of glucose tolerance. Because the reaction of the diabetic patient and the nondiabetic patient to infection appear to be similar, an investigation of the mechanism of carbohydrate intolerance in the normal patient during infection was instituted. Circumstantial evidence obtained through the years suggests that a number of factors might be responsible for abnormalities in carbohydrate metabolism during infection. These factors may be divided into two groups: those involving insulin directly and those involving peripheral pathways of carbohydrate metabolism. Factors involving insulin might be: damage to pancreatic beta cells with a decreased insulin output,[3] enzymatic destruction of insulin,[4] binding of insulin by an antibody,[5] and insulin antagonism at peripheral sites by growth hormone. Factors involving peripheral pathways of carbohydrate metabolism might be glucocorticoid induced gluconeogenesis, increased nonesterified fatty acids, depression of glycogen synthesizing enzymes or other enzyme pathways of carbohydrate metabolism. None of these concepts have been supported by actual measurements of circulating insulin concentrations during infection in the human. Utilizing the radioimmunoassay of serum insulin concentrations[6] as well as the intravenous (IV) glucose tolerance curve, we investigated the impact of acute respiratory tularemia upon carbohydrate metabolism in 7 nondiabetic subjects during a preinfection control period, during maximum allowable infection before institution of therapy and in convalescence 2 weeks later. The magnitude of maximum allowable infection was quantitated by the fever index defined as the degree hours of rectal temperature > 100 F.

The IV glucose tolerance test was used to avoid problems of gastrointestinal absorption, to give a known load of glucose within a brief time limit, and to obtain a well defined initial onset of insulin response. Serial blood samples were analyzed for glucose by a glucose oxidase method. After rapid IV loading, concentrations of glucose decline in a linear fashion when plotted on semilogarithmic paper (Figure 1). Such a straight

* A version has been cleared for publication in Diabetes.

** U. S. Army Medical Unit.

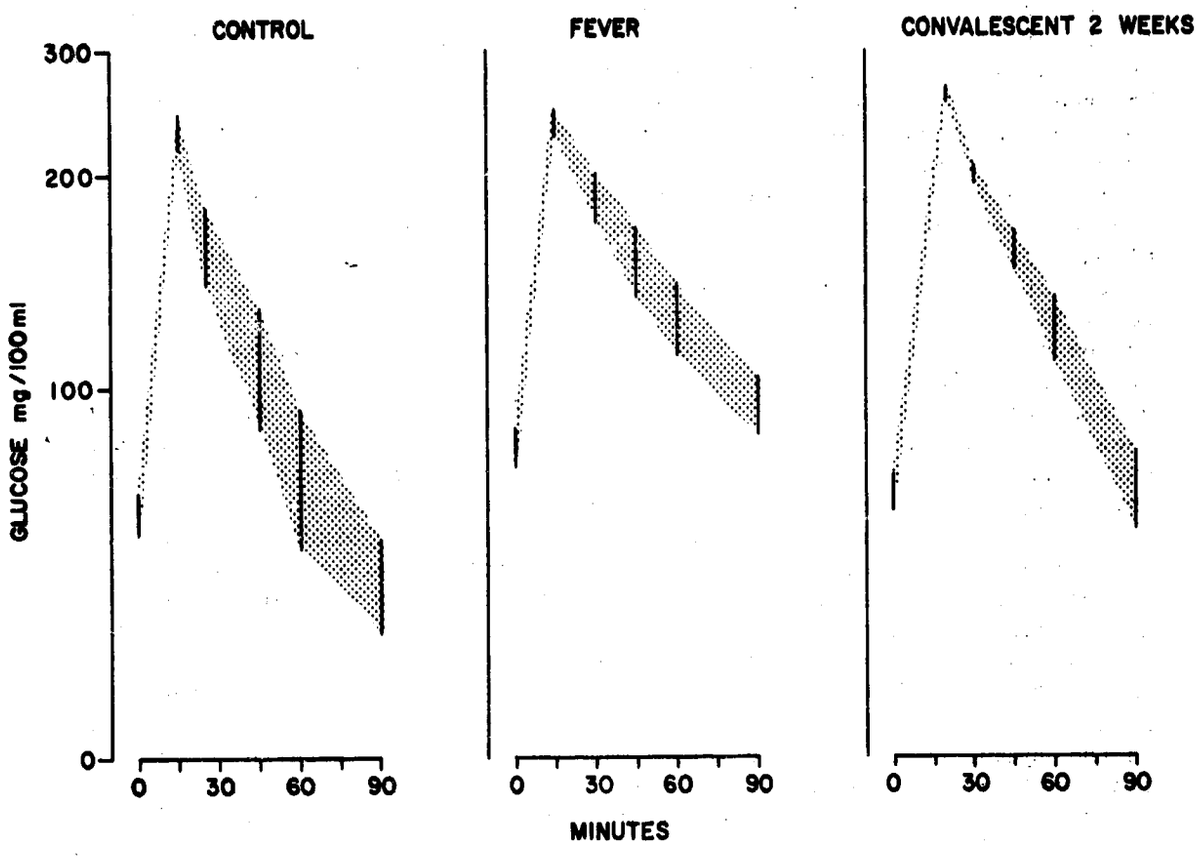

FIGURE I. DISAPPEARANCE RATES OF IV GLUCOSE IN 7 MEN.

line measures glucose disappearance in a dynamic manner; a distinct advantage in detecting suble changes from normal. The semilogarithmic presentation of glucose tolerance was used to calculate the rate of disappearance which was expressed as a value k.

Serum glucose concentrations declined in a linear manner between 15 and 60 min following the infusion. During maximum infection, the descending limb of the glucose tolerance curve became less steep, indicating a delay in glucose disappearance. Although the disappearance rates calculated for the preinfection controls were significantly higher than those obtained during maximum illness all fell within a nondiabetic range. These data suggest that subtle changes in glucose tolerance may occur within 8 hr after the onset of clinical symptoms. Two weeks postinfection, these changes began to return toward normal.

Figure 2 shows values for k ± 1 SE during a control period, during maximum fever and in convalescence. Notice the significant depression during maximum fever, and the return toward normal in convalescence. The bar on the right depicts the k values reported in diabetes. Note that the rate of glucose disappearance in the uninfected diabetic is far less than the normal patient during maximum fever.

Fasting serum glucose concentrations during maximum fever were significantly higher than the values obtained during the preinfection control period. Although the observation of hyperglycemia during acute illness was made nearly a century ago our findings suggest that as early as 8 hr following the onset of clinical symptoms a significant alteration in carbohydrate metabolism has occurred. Two weeks after maximum fever the fasting concentration of glucose had fallen to preinfection control levels suggesting that those factors causing the initial rise were not sustained. In contrast to fasting blood glucose concentrations, the increase of fasting serum insulin concentrations during maximum fever was not significantly higher than the preinfection control.

Following IV glucose administration, serial blood samples were analyzed for insulin by a radioimmunoassay. Insulin concentrations in mU/ml were plotted, a curve drawn and the area defined by this curve used to measure the 3-hr insulin response of the pancreas to the glucose load. This value was expressed in milliunits/ml x minutes (Figure 3).

The insulin response curves during the preinfection control period revealed a rapid onset reaching a peak in 15-30 min, then falling to control values over 2-3 hr, a pattern described by Karam et al.[11] During maximum fever, however, the pattern of insulin output changed. The initial response to the glucose load was followed by a marked delay in the return of elevated serum insulin concentrations to normal. Such a pattern has been described following growth hormone or glucocorticoid administration. In contrast, the pattern of insulin response to a glucose load in obese and maturity onset diabetes is characterized by a delayed initial response which is then followed by a delayed return to normal.

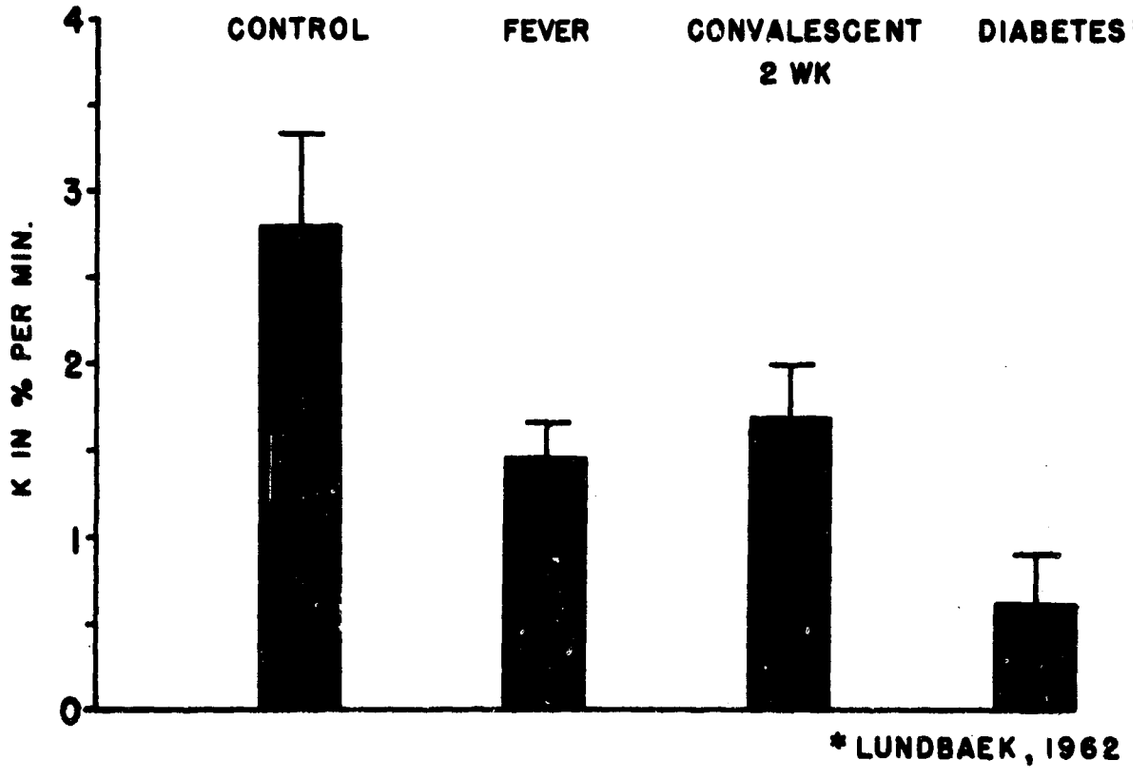

FIGURE 2. DISAPPEARANCE OF BLOOD GLUCOSE IN 7 MEN.

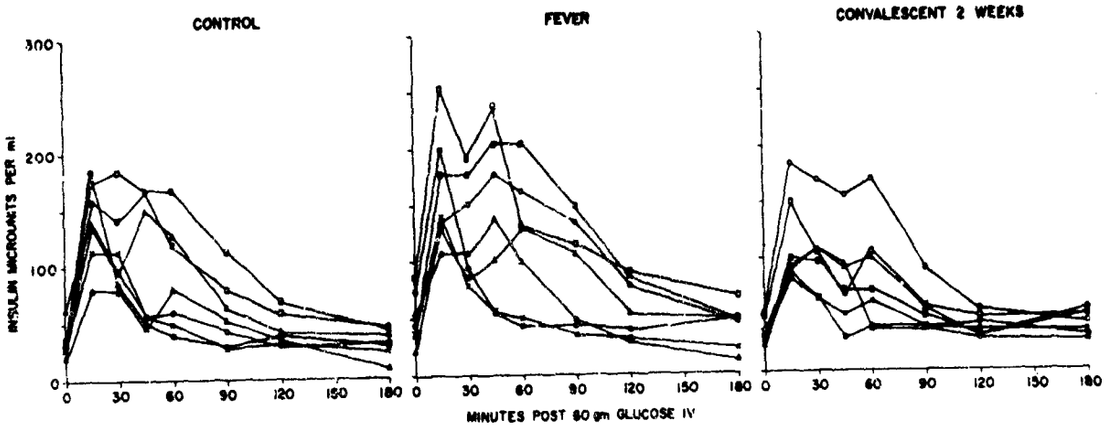

FIGURE 3. SERUM INSULIN RESPONSE TO A GLUCOSE LOAD IN 7 MEN.

The magnitude of the insulin output varied with each individual but the increase in this response during infection remained relatively constant for each individual. The difference was significant at the 2% level. This suggested that the stimulus to increased insulin output during infection had affected all individuals to the same degree.

Because the insulin response during infection appeared to increase while the rate of serum glucose disappearance decreased, we attempted to find some relationship between the fever index, insulin response, and glucose tolerance abnormalities. Of the many correlations made, only 3 were significant (Figure 4).

The fever index on the day of maximum allowable illness correlated directly with the relative increase in insulin output shown to the left. At the same time the rate of disappearance of serum glucose correlated inversely with the fever index. Finally, the rate of glucose disappearance was inversely related to the insulin response following the glucose load on the day of maximum allowable illness. These findings may be interpreted in several ways. Fever was associated with an increased insulin output and a depression of glucose tolerance; therefore, the relationship between insulin and glucose may be the result of fever alone. Secondly changes in fever, glucose, and insulin may be related to a common alteration in the host during infection, and the relationship among the 3 is fortuitous. Finally, since elevations in blood glucose concentrations are known to stimulate insulin output, the increased insulin response may be secondary to a primary abnormality in carbohydrate metabolism that is somehow related to fever. In one asymptomatic patient a persistence of increased insulin output and a flattened glucose tolerance curve was seen 2 weeks postinfection. This suggests that other factors besides fever may play a role.

Many factors affecting peripheral pathways of carbohydrate metabolism during a generalized infection have been investigated concurrently during previous studies of acute respiratory tularemia in man. Such factors can be classified as hormonal and nonhormonal.

Beisel and his workers[8/] investigated adrenocorticoid alterations during a generalized infection and found an increase in urinary cortisol excretion. Although cortisol administration has been shown by other investigators to increase insulin output, the dosages required were 4-5 times those used by Beisel to duplicate the cortisol excretion during maximum infection. Cortisol has also been shown to increase whole blood pyruvate levels following glucose loading. Alterations in thyroid hormone concentrations in the human during acute respiratory tularemia have been studied by myself. Briefly, thyroid hormone output decreases, the protein bound iodine falls and the unbound physiologically active portion decreases. As these changes were barely significant, their effect upon carbohydrate metabolism would probably be of little importance.

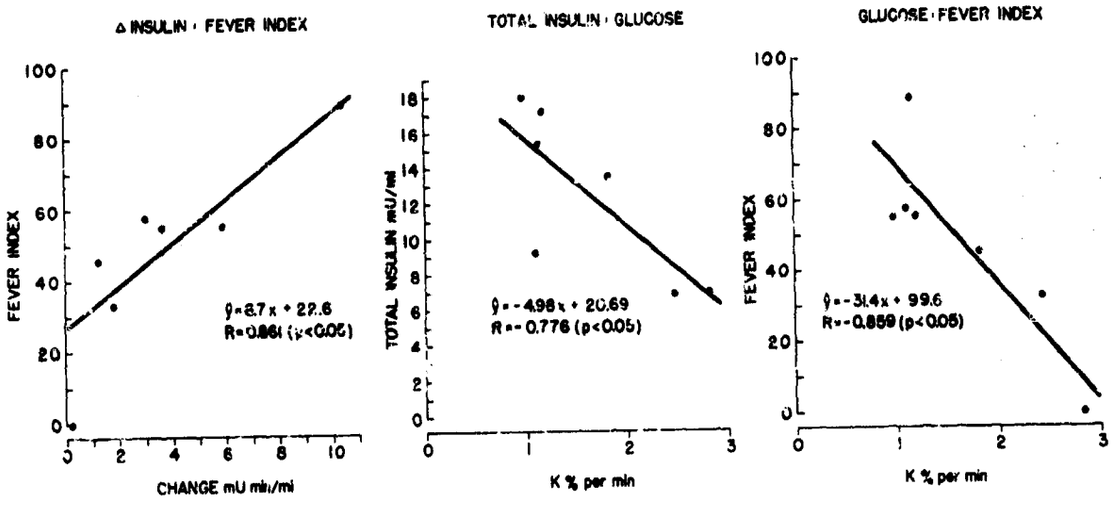

FIGURE 4. INTERRELATIONSHIPS OF INSULIN OUTPUT, FRACTIONAL TURNOVER OF GLUCOSE AND FEVER DURING INFECTION IN 7 MEN.

In order to study nonhormonal alterations in carbohydrate metabolism, the human red cell was chosen. This model has the advantage of insulin insensitivity and two well defined paths of carbohydrate metabolism: the pentose shunt and the Embden Myerhoff pathway. During maximum fever, Beisel[9] demonstrated a depression of C^{14}-labeled glucose metabolism by the pentose shunt. These studies were extended by myself to 2 enzymes of this pathway glucose-6-phosphate dehydrogenase and 6-phospho-gluco-dehydrogenase. These were also depressed slightly during maximum fever.

Finally, whole blood pyruvate concentrations were studied and found to increase during maximum infection. This finding suggests that metabolism of pyruvate may be depressed as well.

The peripheral changes seen during tularemia are minimal but when taken together, a combination of increased adrenal hormone output superimposed upon suppression of various enzyme systems could result in hyperglycemia and an abnormal glucose tolerance curve. Under these circumstances, the increased insulin output seen during infection may well be a secondary phenomenon related to the increased blood sugar concentration. Other factors such as the role of growth hormone and nonesterified fatty acids remain to be investigated.

The deterioration of glucose tolerance during infection in man is accompanied by an increased insulin response to glucose loading. These findings are compatible with a transient peripheral inhibition of insulin activity during the period of maximum fever.

SUMMARY

Serum insulin concentrations were measured serially in 7 nondiabetic subjects following a rapid intravenous (IV) glucose load during a preinfection control period, early clinical respiratory tularemia, and again in convalescence following therapy. Within 24 hr of onset of clinical illness the rate of glucose disappearance from the blood had diminished significantly. In contrast, there was a brisk onset of insulin response which reached higher peak concentrations and fell more slowly than the response observed during the preinfection control period. The pattern during clinical illness was different from that described after IV glucose loading in maturity onset diabetes or obesity, and may have been influenced by glucocorticoid excess. The magnitude of insulin response during clinical illness was significantly increased and was directly related to height of fever. In contrast, the rate of glucose disappearance was inversely related to fever. The inverse relationship of the magnitude of insulin output to the rate of glucose disappearance suggested a peripheral inhibition of insulin action during infection. Although fever may have played a role during acute illness, the persistence of an abnormal insulin response in one patient during convalescence suggested that this change was not dependent upon fever alone.

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